ADVANCEMENT OF OVULATION IN YELLOWTAIL FLOUNDER (Pleuronecte ferrugineus) USING GONADOTROPIC HORMONE-RELEASING HORMONE ANALOGUE (GnRHa)

STEPHEN PAUL BETTLES

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Advancement of ovulation in yellowtail flounder (*Pleuronectes ferrugineus*) using gonadotropic hormone-releasing hormone analogue (GnRHa)

By

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Abstract

Yellowtail flounder (*Pleuronectes ferrugineus*) are being considered for aquacultural development and in order to maximize productivity within a hatchery, a thorough knowledge of the reproductive cycle and how it may be manipulated, must be attained.

Ovarian biopsies, using polyethylene cannulae, were used to follow the annual oocyte growth pattern in yellowtail flounder. Vitellogenesis, as measured by an increase in both oocyte diameter and opacity, began in October-November, and progressively continued until the spawning season, between June and August. The use of cannulae for ovarian biopsies in yellowtail flounder was validated by assessing homogeneity of oocyte sizes throughout the ovary, indicating that oocytes from the anterior ⅓ of the ovary were representative of the entire left and right ovaries.

The capacity to advance the yellowtail flounder spawning season using gonadotropic hormone-releasing hormone analogue ([D-Ala^6,Pro^9-NHEt]GnRHa) was studied in maturing females by administering the hormone in three separate spawning trials, four months, two months and two weeks prior to the natural spawning season. From ovarian biopsies taken at time of treatment, it was found that the spawning response was dependant upon oocyte size reaching approximately 400μm before final egg maturation, and ovulation, could be induced. In addition, the response to GnRHa treatment was quicker, more uniform, and better synchronized closer to the naturall spawning season, with 25%, 100% and 100% of the

females spawning in each group, respectively, and the mean number of days to first ovulation being 25, 20, and 14 days, respectively.

Egg quality was measured by morphological criteria of unfertilized eggs (viability rates or the percentage of potentially fertilizable eggs), fertilization rates, and hatch rates and were high and similar in each group. Viability rates for each group and the control group were 87.1%, 83.0%, 89.9% and 82.9%, respectively. Fertilization rates were 71.0%, 63.7%, 70.7% and 54.6%, whereas hatch rates were 79.2%, 85.4%, 86.8%, and 80.5%, respectively. Advancement of spawning by GnRHa had no deleterious effects on egg quality when compared to spontaneously ovulating control fish.

Acknowledgments

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[1] Crim and Bettles (1997); excerpt written by the latter author.

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Introduction

1.1 Yellowtail Flounder Life History

The yellowtail flounder (*Pleuronectes ferrugineus* Storer 1839) has an extensive range throughout most of the continental shelf waters of the Northwest Atlantic. The southerly limit of its range is Chesapeake Bay and it extends northward to the Strait of Belle Isle (Bigelow and Schroeder, 1953; Scott and Scott, 1988). There are five main stocks of yellowtail flounder throughout these waters which include the New England, the Georges Bank, the Cape Cod, the Scotian Shelf and the Grand Banks stocks, and dispersion between these distinct stocks is generally perceived to be minimal (Royce et al., 1959). Since the mid-1960's, yellowtail flounder has been a commercially important species and increasing fishing pressure has reduced their numbers significantly in many areas (Howell and Kesler, 1977; Walsh et al., 1995).

A member of the family Pleuronectidae, the yellowtail flounder is right-eyed, meaning that during metamorphosis the left eye migrates to the right as the fish settles on its left side. According to Scott and Scott (1988), they prefer temperatures between 2-6 °C but have a much wider tolerance from sub-zero temperatures to the mid to high teens in the laboratory (pers. obs.) and prefer salinities of 32-33ppt. Maximum sizes recorded range from 59.0cm (Scott and Scott, 1988) to 62.7cm (Anonymous, 1932). The yellowtail flounder has a recorded maximum longevity of 12 years with maturity occurring at 4 and 5 years for Grand

Banks stocks of males and females, respectively (Pitt, 1970), and 2 and 3 years for males and females, respectively, of the New England stocks (Lux and Nichy, 1969). A mature female may produce between 350,000 and 4,570,000 eggs in one spawning season (Pitt, 1971), however fecundity can be directly correlated with fish length, age and size of ovary (Howell and Kesler, 1977). Newly spawned eggs average in diameter between 750 and 900μm, are pelagic and do not contain an oil globule (Yevseyenko and Nevinskiy, 1982).

As new and alternative species are being sought for introduction into aquaculture, the yellowtail flounder seems a promising choice since it has a high quality flesh and markets are already established (Bettles, 1996). Prior to the beginning of an aquaculture endeavour, the reproductive biology of the yellowtail flounder must be investigated, and many fundamental questions must be answered before the fish can be reproduced in captive conditions on a commercial basis. There are several reports pertaining to the reproductive cycle of female yellowtail flounder (Scott, 1947; Howell, 1983; Zamarro, 1991). The timing of spawning shows a latitudinal cline, occurring between April and June in the southern limits of Chesapeake Bay (Bigelow and Schroeder, 1953) and between June and August in the northern limits of the Strait of Belle Isle (Pitt, 1970). Yellowtail flounder conform to the description of annual group-synchronous spawners (Wallace and Selman, 1981), meaning a clutch of oocytes are synchronously recruited into vitellogenesis annually, while a second population remains previtellogenic and will be spawned in subsequent years. Over the duration of the spawning season, 14-22 egg batches are produced (Manning, 1997).

1.2 GnRHa Induction of Spawning

The use of GnRHa in aquaculture is gaining widespread acceptance over the classical hormone therapies such as steroids, or pituitary preparations. GnRHa serves several purposes such as, to advance the spawning season, to initiate the processes of final maturation of gametes, or to synchronize the spawning of broodstock. Under captive conditions, fish often develop mature gonads (and gametes) but fail to undergo the processes of final maturation and spawning, due to an insufficient surge of gonadotropic hormone (GtH) from the pituitary. Thus, females may develop vitellogenic oocytes yet remain incapable of ovulating.

The release of GtH from the fish pituitary is under direct control of the hypothalamus via neuronal contact. Governed by a dual hormonal system, GtH release is inhibited by the catecholamine dopamine and stimulated by GnRH (Peter et al., 1988). Under appropriate environmental cues, the balance of these two hormones is shifted, eliciting the GtH response. To artificially induce spawning in many freshwater fish, such as the cyprinids, a dopamine antagonist (e.g., pimozide, metoclopramide, domperidone, or haloperidol), in conjunction with GnRH analogues is necessary. For example, neither mGnRHa nor pimozide alone evoked a spawning response in the catfish, *Clarias batrachus*, but mGnRHa combined with pimozide produced high rates of ovulation, fertilization and hatching of normal larvae (Manickam and Joy, 1989). Carp do not spontaneously spawn in captivity but within 19-21 hours of implanting maturing female common carp, *Cyprinus carpio*, with either sGnRHa and metoclopramide, or carp pituitary extract, ovulated eggs were released (Drori et al., 1994). Circulating levels of GtH and estradiol-17ß increased reaching a peak 3 hours after ovulation,

whereas the levels of 17α-20ß-dihydroxy-4-pregnen-3-one began to increase within 7 hours of treatment, concomitant with germinal vesicle breakdown.

The use of dopamine antagonists (= Linpe method), for spawning captive fish has several advantages (Peter et al., 1993): 1) it produces complete or consistently high rates of ovulation within groups of broodstock, 2) times to ovulation after treatment are short and predictable, 3) high egg fertilization and viability rates are usually obtained, and 4) it has little or no effects on subsequent reproductive cycles.

Not all fish species seem to require dopamine antagonists with GnRHa to induce spawning. For example, winter flounder, *Pleuronectes americanus* (Harmin and Crim, 1992; Harmin et al., 1995), yellowtail flounder, *Pleuronectes ferrugineus* (Larsson et al., 1997), sea bass, *Lates calcarifer* (Garcia, 1989b,c), Atlantic salmon, *Salmo salar* (Crim et al., 1986; Taranger et al., 1992), and channel catfish, *Ictalurus punctatus* (Busch and Steeby, 1990) spawn spontaneously or ovulate eggs for stripping in response to GnRHa alone. For these species the dopaminergic inhibitory tone of GnRH action is either less important than it is in cyprinids (Breton et al., 1990), or dopaminergic inhibition may be absent. For example, when female juvenile and adult Atlantic croaker, *Micropogonias undulatus*, were treated with mGnRHa in combination with various dopamine antagonists, none of the combinations increased circulating levels of GtH above those treated with mGnRHa alone. Dopamine agonists such as bromocryptine or apomorphine also had little or no effect on the mGnRHa response, suggesting that there is a lack of dopaminergic inhibition for GtH secretion in the croaker (Copeland and Thomas, 1989).

1.2.1 Administration of GnRH

GnRHa can be administered in either of two ways, *acutely* or *chronically*, for short- or long-term stimulation of GtH release from the pituitary gland. For acute treatments, GnRHa with or without a dopamine antagonist, is given as a single injection to maturing fish close to spawning to either initiate final oocyte maturation in females or enhance spermiation in males. Injections are usually administered interperitoneally or intramuscularly by means of a saline vehicle (Fermin, 1991; Glubokov et al., 1991) and have occasionally been given intracranially (Busch and Steeby, 1990). Some fish may require more than a single GnRHa injection to initiate the final maturation process, and several treatments over a period of days to weeks may be required (Slater et al., 1995; Alvariño et al., 1992; Glubokov et al., 1991; Sanborn and Misitano, 1991).

For fish sensitive to handling stress, or for treatment of fish several times, the injection method is not practical. Therefore non-invasive delivery systems or sustained release delivery systems are required (Thomas and Arnold, 1993). Thomas and Boyd (1989) fed mGnRHa to spotted seatrout, *Cynoscion nebulosus* - a sciaenid fish highly susceptible to handling stress. Mature females were given one or two dead shrimp, injected with mGnRHa to yield 1.0-2.5 mg/kg fish body weight. Consequently, the number of shrimp eaten per female and thus the dose of the hormone could not be ascertained. Ovulation and spawning commenced within 32-38 hours after feeding and high egg fertilization and hatch rates were attained. Since none of the controls spawned during this period, it was concluded that oral administration of GnRHa is an effective and non-invasive means to induce spawning in this

species. However, this method has several drawbacks: 1) controlling the hormone dosage each fish receives is difficult, 2) it is applicable only for fish that continue feeding during the reproductive period, and 3) either high hormone doses or GnRH analogues that resist gastric degradation must be used. McLean et al. (1991) intubated coho salmon, *Oncorhynchus kisutch* with various doses of mGnRH or mGnRHa to assess the permeability of the gut to orally administered GnRH. From blood samples taken over a 6 hour period, an elevation in plasma GtH levels was found within 30 minutes of oral delivery with the analogue being absorbed more rapidly and in greater quantities than the native peptide. In a similar study, sGnRHa was intubated orally or anally in African catfish, *C. gariepinus*, rainbow trout, *Oncorhynchus mykiss*, and the agastric common carp, *C. carpio* (Breton et al., 1995). In the carp, the same GtH elevation was attained despite the route of administration, whereas in the catfish, GtH elevation was stimulated by anal intubation only. Thus it was concluded sGnRHa is degraded by pepsin and acidic pH in the stomach. To overcome this drawback and to reduce the dose of hormone required, enteric microcapsules containing sGnRHa were developed and were successful in elevating GtH levels and inducing spawning in all three species with doses as low as 5 μg/kg sGnRHa. The addition of GnRHa to feed to form "maturation diets", holds promise for developing noninvasive GnRHa delivery systems, that reduces both fish handling and farm labour (Breton et al., 1995).

Chronic or sustained GnRHa administration requires either multiple injections or delivery vehicles that slowly release the hormone over prolonged periods, since the length of exposure to GnRHa may be more important than the dose to achieve GtH release (Almendras et al.,

1988). This method can be used to advance final maturation by accelerating vitellogenesis, or it can provide continuous stimulation over a series of consecutive spawning events as for batch spawners, such as the gilthead sea bream (Zohar et al., 1995; Zohar et al., 1989), sea bass (Almendras et al., 1988; Harvey et al., 1985), and yellowtail flounder (Larsson et al., 1997). Cholesterol pellets containing GnRHa are commonly used for sustained hormone release. The rate of release of GnRHa from the pellets can be controlled by combining cholesterol with cellulose (Sherwood et al., 1988). *In vitro* studies have shown that at 9 °C, pellets of cholesterol/cellulose (4:1 *w/w*) release 79% of the hormone within 6 hours and 94% within 12 hours. For 100% cholesterol pellets, 50% GnRHa is released between 100-600 hours (Carolsfeld et al., 1988).

The use of cholesterol as a releasing medium has been criticized for practical use since it is a precursor for steroids and the pellets are not biodegradable (Breton et al., 1990). Subsequently, several biodegradable sustained release delivery systems have been developed. Recently, Mylonas et al. (1995) developed polyanhydride microspheres containing mGnRHa, based on a biodegradable copolymer of fatty acid dimer and sebacic acid. The microspheres maintained elevated plasma levels of GnRHa in striped bass, *Morone saxitalis*, for at least 8 weeks, and induced ovulation within 11 days. When injected into mature female white bass (Mylonas et al., 1996) and Atlantic salmon (Mylonas et al., 1995), ovulation occurred within 35-82 hours, and 15 days, respectively. Other biodegradable implants have been made from the copolymer of polylactic-polyglycolic acid (PLGA) and have been shown to maintain elevated levels of GtH in maturing female sea bream for at least 10 days (Zohar et al., 1995).

The use of GnRHa to induce spawning may affect gamete (primarily egg) quality, yet studies relating to this subject are sparse. In brown trout, *Salmo trutta*, injected with various doses of mGnRHa, significantly lower egg fertilization rates were observed in the group given the highest (10 µg/kg) dose (Mylonas et al., 1992). However, reduced fertilization rates were not attributed to dose or treatment, but rather time to ovulation after initial injection. It was concluded that fish induced to spawn early in the season had insufficient time to resume meiotic maturation in the oocytes before being ovulated and therefore the eggs were incapable of being fertilized. In a study on early maturing Atlantic salmon subjected to various doses of mGnRHa, Taranger et al. (1992) found no differences in egg and larval survival at doses of 1 and 10 µg/kg BW. However, at doses of 100 µg/kg BW, high mortalities until eyed-stage were observed.

Other problems that may arise from inducing early spawning are the potential changes in physical and chemical parameters of gamete quality. Ako et al. (1994) compared egg quality between hormonally induced and naturally spawned milkfish, *Chanos chanos*. The hormonally induced spawners produced higher numbers of eggs, although they were smaller and had lower fertilization rates. All fatty acids, except 18:1*n*-9, 18:3*n*-3, 22:1*n*-11, and 22:6*n*-3, were significantly lower. Conversely, naturally spawned eggs had a higher protein content and a higher amino acid content (except methionine, leucine, and phenylalanine), while nonessential amino acids were the same for both groups.

1.3 Assessment of Reproductive Maturity

All published studies to date on the female yellowtail flounder reproductive cycle have been based on histological sections of ovaries from fish taken by commercial trawl fisheries. This study represents the first use of the technique of ovarian biopsies (by cannulation) in yellowtail flounder, to evaluate oocyte growth throughout the annual reproductive cycle using the same group of individuals.

Removal of oocytes by means of polyethylene cannulae has been previously used for observing the reproductive status of females *in vivo* without the need for sacrificing fish (Shehadeh et al., 1973; Garcia, 1988a). This is of utmost importance in an aquacultural setting where broodstock are both limited and valuable. Ovarian biopsies are especially useful for accurately determining the reproductive status of individual females prior to the administration of hormonal therapy.

1.4 Objectives

The primary objectives of this thesis were to determine the annual oocyte growth pattern of yellowtail flounder held under captive conditions, and to investigate the success of using gonadotropic hormone-releasing hormone analogue (GnRHa) to advance ovulation in females, and its subsequent effect on egg quality.

Chapter 2

Methods and Materials

2.1 Environmental Conditions

Most yellowtail flounder were collected in November 1994 using SCUBA, from Witless Bay (47° 15' N, 52° 45' W) and Conception Bay (47° 33' N, 53° 03' W). Fish were maintained at the Ocean Sciences Centre, Logy Bay, Newfoundland in 2000L stock tanks and subjected to ambient photoperiod. Several weeks to months prior to the beginning of each trial, fish were moved to 400L experimental tanks. Temperature of flow-through seawater (2-9 °C) was maintained by mixing ambient seawater with heated/chilled seawater (Fig. 2.1). Simulated ambient photoperiod was provided by an instant on/off 60W incandescent bulb suspended 80cm above the water surface (75-190 Lux at surface). Fish were fed moist marine pellets at 2% body weight twice weekly prior to, and occasionally during spawning, and three times weekly for three months after spawning.

2.2 Experimental Animals

Eight females were selected for each of three GnRHa spawning trials as well as the control groups, according to size (weight: 390 ± 117g, length: 33 ± 2cm), gonadal development and sexual maturity. Only fish possessing well developed gonads, extending at least half the distance from the abdomen to the caudal peduncle, were used (Maturity stage 2+ to 3: as described by Sanborn and Misitano, 1991). The estimated age of females was 4-6

years (Pitt, 1974). At least 3 mature males were also added to each tank, to provide milt for egg fertilizations. All fish were individually identified (Table 2.1) with a passive integrated transponder tag (PIT tag: Destron IDI, Boulder, Colorado) implanted into the right-dorsal musculature. During spawning, females were also identified with the use of coloured beads attached by silk thread to their right opercula.

2.3 Ovarian Biopsies

Ovarian biopsies were performed to assess the maturity stage of females either monthly throughout the annual reproductive cycle, or immediately prior to hormone treatment in the GnRHa experiments. Females were anaesthetized with 0.25mL/L 2-phenoxyethanol, the weight and length measured, and oocytes removed by cannulation. A polyethylene cannula (inner dia.=1.19mm, outer dia.=1.70mm, length ≈ 15.0cm) was inserted into the anterior ⅓ of the right (eyed-side) ovary via the ovipore and oocytes were aspirated using oral suction (Fig. 2.2). Slight pressure on the external surface of the fish was sufficient to determine the location of the cannula within the ovary. Unless otherwise stated throughout, 'oocytes' refers to vitellogenic oocytes.

Oocytes were immediately transferred into a chilled 1% formalin, 0.6% saline preservative solution, refrigerated, and the follicular oocyte diameter measured (n = 50-100 per sample) after 24 hours, using a stereo microscope (at 40×) fitted with an ocular micrometer. According to Shehadeh et al. (1973), this preservative has no significant effect on oocyte volumes for grey mullet (*Mugil cephalus*). To test this assumption for yellowtail

flounder oocytes, five fish were cannulated and their oocytes kept on ice, in the cannula, for <5 min. Diameter measurements (n = 20 per fish) were then recorded within 2 min after transferring to preservative, and at 15min, 1h, 1d, 2wk and 4wk thereafter. All oocyte diameters have been adjusted for preservative effects and thus represent the 'live' measurement

Oocytes removed by cannulation must be representative of the maturational state of the entire ovary (Garcia, 1989a). To determine if oocytes taken by cannulation were characteristic of *in vivo* oocytes, samples of oocytes were withdrawn from the anterior ⅓ of the ovaries of four fish, after which the fish were killed. Ovaries were then excised and placed in preservative for 24h. Oocytes were carefully removed from the lumen near the sampling site of the cannula and their diameters were recorded.

To determine if cannulated oocytes from the anterior ⅓ of the right ovary were representative of the entire ovary, excised right ovaries from five females were divided into three equal length portions, stored in preservative for 24h, and >100 oocytes carefully removed from the lumen of each portion. Similarly, to determine if oocytes from the right ovary were comparable to those from the left, both left and right ovaries were removed from three fish, stored in preservative for 24h, and the diameters of 50 oocytes from the anterior ⅓ of each ovary were measured.

2.4 GnRHa Hormone Pellet Preparation

Gonadotropic hormone-releasing hormone analogue [D-Ala^6,Pro^9-NHEt]LHRH (Syndel Laboratories, Vancouver, BC), at a concentration of 100µg/pellet (200µg/kg fish body weight), was embedded in 2×4mm slow-release cholesterol pellets and injected into the right-dorsal musculature (Crim et al., 1983). Control fish received blank cholesterol pellets without GnRHa. All pellets were stored in a desiccating chamber at 4°C, until required.

2.5 Egg Quality Evaluation

Beginning several days after GnRHa treatment, fish were assessed daily for signs of ovulation. Yellowtail flounder, similar to other flatfish, have a pronounced swelling of the ovaries, primarily visible above the right ovary, at later stages of maturation. Shortly before ovulation, ovaries become softened due to hydration of the oocytes. Ovulated eggs from fish were stripped into a chilled plastic beaker on a daily basis until no more batches could be attained. Eggs volumes were determined in graduated cylinders and any egg volumes less than 3mL were considered residual from the previous ovulation, and discarded.

The quality of egg batches was assessed according to egg viability rates, fertilization rates and hatch rates. Viability rates indicate the percentage of unfertilized eggs which may have the potential to be fertilized, based solely on morphological characteristics. A viable yellowtail flounder egg is considered spherical, clear, positively buoyant and lacks a perivitelline space (Larrson et al., 1997). Viability rates were determined for each batch from the first 100 eggs examined of each triplicate 150µL aliquot of unfertilized eggs placed in

seawater. To determine fertilization rates, triplicate 75μL aliquots (approx. 100-150 eggs) from each batch were 'dry' fertilized in Petri dishes, with 5μL of freshly collected milt pooled from 2 males, where an undiluted volume of 5μL of milt is considered sperm supersaturation (Clearwater, 1997). Prior to and after the pooling of milt samples, sperm motility (after seawater activation) was verified under the microscope. Only milt with >75% sperm activation was used. Yellowtail flounder milt is present year-round in the sperm duct of some males, at least in small amounts (Clearwater, 1997), thus GnRHa treatment of males was not necessary. Egg fertilization rates were recorded when the 4-8 cell-cleavage stage was reached, after a six hour incubation period at 6°C, and expressed as a percentage of the number of fertilized eggs per total number of eggs incubated.

Seawater used for both the activation of sperm and in the Petri dishes was 1μm filtered, UV sterilized, and supplemented with 0.06% penicillin G and 0.1% streptomycin sulfate (Sigma Chemicals, Illinois) to inhibit bacterial and fungal growth, respectively. Seawater was exchanged every 2-3 days and dead eggs removed, then after 8 to 12 days of incubation at 6°C, hatched larvae were removed and counted. The hatching rates were calculated as the percentage of fertilized eggs to reach hatch.

2.6 Statistical Analysis

Data were analysed using the SAS statistical software package (SAS Institute Inc., Cary, NC). All calculated values are expressed as the mean ± 1 standard deviation (SD). Oocyte size differences between anterior ⅓ and the entire ovary were compared using one-way

analysis of variance (ANOVA) followed by Student-Newman Keuls, Tukey's and GT2 multiple comparisons of means. Oocyte sizes between the left and right ovaries were compared using ANOVA. Egg quality data (viability, fertilization and hatch rates) for each GnRH trial were subjected to a mixed hierarchical ANOVA (Zar, 1982), followed by GT2 comparison of means (Sokal and Rohlf, 1996). Levels included treatment (n = 4), individual spawning fish (n = 21), individual egg batches (n = 435), and replicates (n = 3 per batch). Tolerance for a Type I error was set at a level of 0.05. Residuals were also analysed for normality by visual inspection of residuals plotted against predicted values, normal probability plots, and histograms and of residuals. All residuals were normally distributed unless otherwise stated.

Figure 2.1 Seasonal variation in average monthly temperature (°C). Values were recorded daily as the mean of daily minimum and maximum. Vertical bars = ±SD

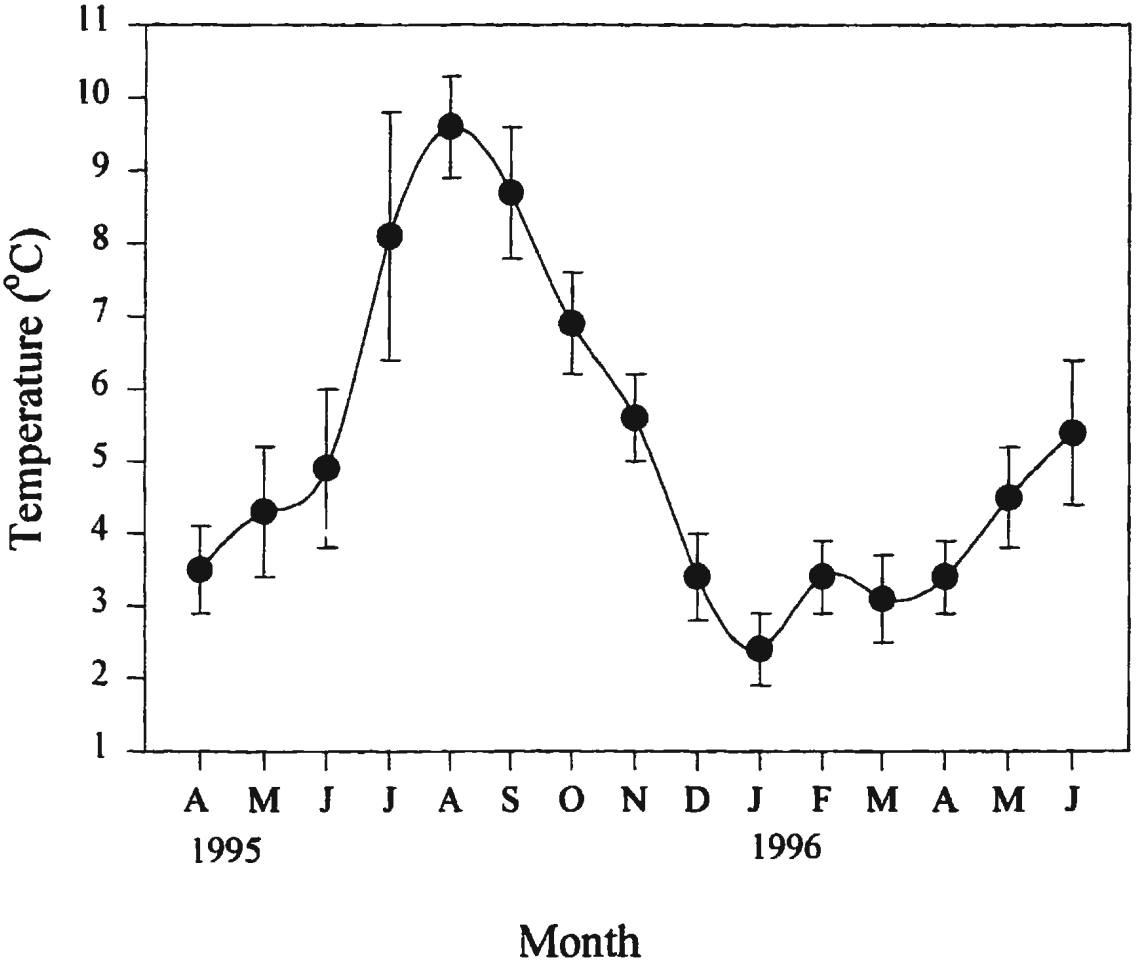

Temperature (°C)
11
10
9
8
7
6
5
4
3
2
1
A M J J A S O N D J F M A M J
1995
1996
Month

Table 2.1 Identification, initial weight and length, and capture location of individual fish used in each experiment (- information not available)

Experiment	Fish ID		Capture Date	Capture Location	L (cm)	W (g)
Annual Cycle	224B0F	BB	Nov-94	Witless Bay	31.0	306
(four month control)	165F5A	BG	-	-	29.0	396
	21400D	GG	Nov-94	Witless Bay	32.5	299
	036D54	RG		-	30.0	252
	355941	RO	Nov-94	Witless Bay	32.0	382
	16520B	YG	-	-	34.0	589
	0E7273	YO	-	-	35.0	426
	745B21	YY	Nov-94	Witless Bay	34.0	424
Implanted 4 months ahead	011D55	6O	Jul-95	Kelly's Island. CB	33.0	441
of normal spawning	39311F	6B	Nov-94	Witless Bay	29.0	343
	413479	6G	Nov-94	Witless Bay	34.0	441
	077A5E	6P	Nov-94	Witless Bay	33.0	401
	11570B	6R	Nov-94	Witless Bay	31.5	415
	081C6C	6V	Jul-95	Kelly's Island. CB	38.0	858
	0F085C	6W	Nov-94	Witless Bay	31.5	387
	457A28	6Y	Nov-94	Witless Bay	32.0	449
Implanted 2 months ahead	35750F	2B	Nov-94	Witless Bay	34.5	356
of normal spawning	76146B	2G	Nov-94	Witless Bay	36.0	424
	043230	2O	-	-	37.0	504
	0E7E67	2P	-	-	33.5	365
	0F352F	2R	-	-	29.5	225
	146445	2V	-	-	32.0	325
	0D2774	2W	Nov-94	Witless Bay	31.0	341
	154026	2Y	Nov-94	Witless Bay	35.0	496
Implanted 2 weeks ahead	0E617B	4B	Nov-94	Witless Bay	32.0	279
of normal spawning	0E023E	4G	-	-	31.0	298
	353C55	4O	-	-	33.0	355
	04311F	4P	1993	DFO	36.0	587
	091456	4R	-	-	31.0	280
	34376B	4V	1993	DFO	33.0	558
	343A55	4W	Nov-94	Witless Bay	31.5	347
	784D5C	4Y	Nov-94	Witless Bay	34.0	410
Control (2 month and 2 week)	093F2B	3B	Nov-94	Witless Bay	31.0	287
	097B6F	3G	Nov-94	Witless Bay	29.0	276
	1E5C5F	3O	-	-	34.5	432
	7D3424	3P	Nov-94	Witless Bay	31.5	422
	12637E	3R	Nov-94	Witless Bay	32.5	309
	110F53	3V	Nov-94	Witless Bay	34.0	373
	460201	3W	Nov-94	Witless Bay	30.0	269
	117270	3Y	Nov-94	Witless Bay	30.5	264

DFO - Caught by Department of Fisheries and Oceans trawls on Grand Banks

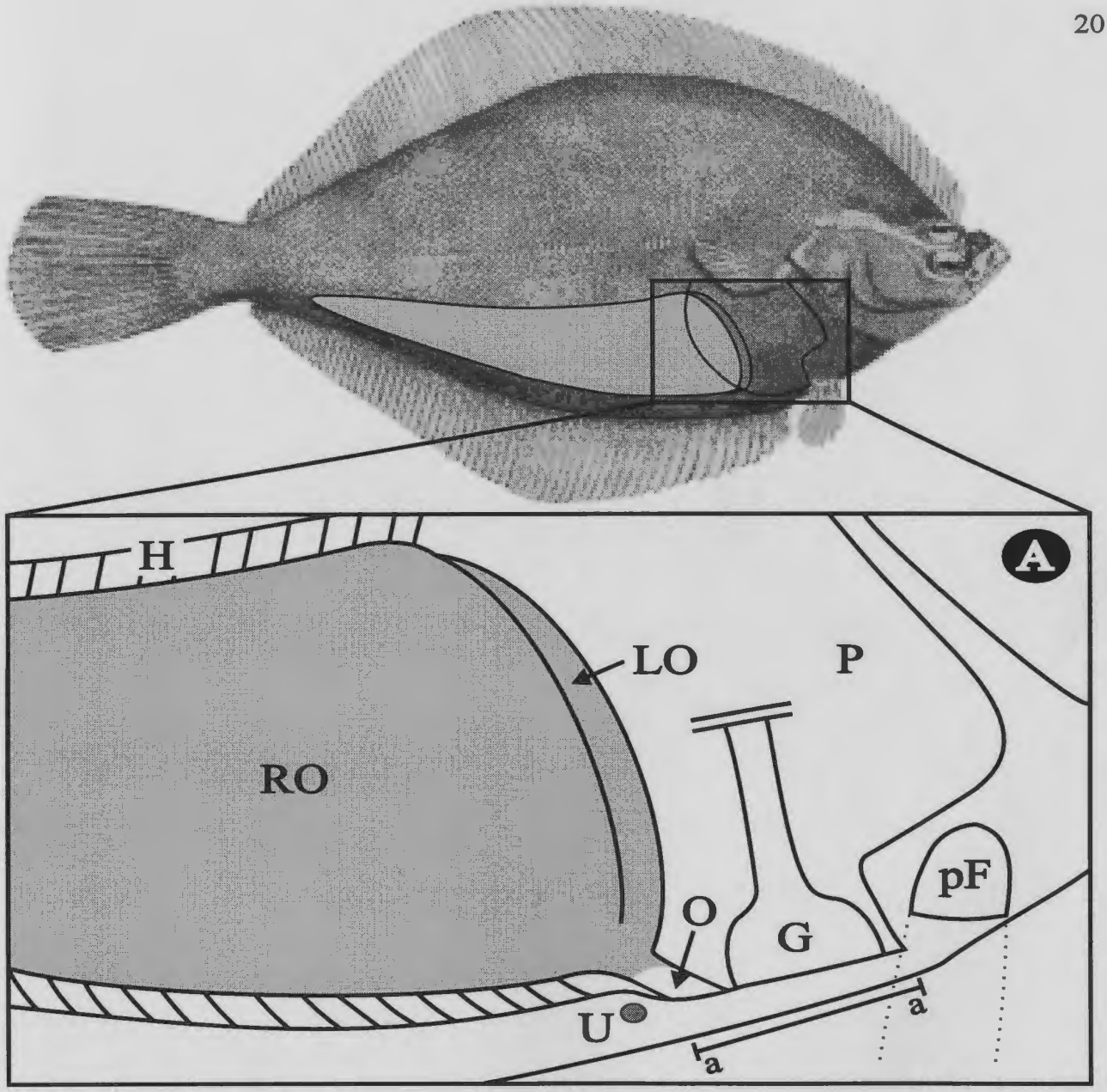
Figure 2.2 a) location of ovaries and the gut in yellowtail flounder.

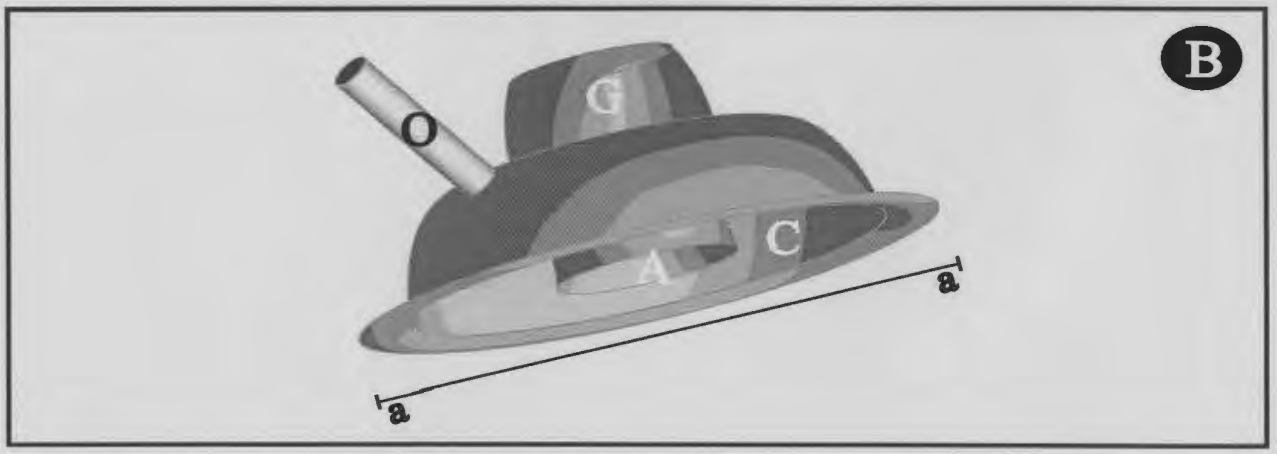
b) section through the cloaca (a—a).

A - anus; **C** - cloaca; **G** - gut; **H** - haemal spines; **LO** - left ovary;

O - oviduct; **P** - peritoneum; **pF** - pelvic fin (... fin removed);

RO - right ovary; **U** - urinary pore


H
A
LO
P
RO
pF
O
G
U
a
a


B
G
O
C
A
a
a

Chapter 3

Validation of ovarian cannulation for monitoring oocyte growth in yellowtail flounder

3.1 Introduction

Yellowtail flounder are batch spawning teleosts found throughout the waters of the Northwest Atlantic. Of several studies that have addressed the reproductive cycle of female yellowtail flounder, one of the more comprehensive studies by Howell (1983) described the seasonal changes in yellowtail flounder ovaries histologically. Similarly, Zamarro (1991) histologically examined the ovaries of mature females to determine the batch fecundity and spawning frequencies. In each study, fish were derived from commercial trawls. Recently, yellowtail flounder have been considered potential candidates for coldwater aquaculture and further studies of their reproductive development are warranted. However, due to the limited availability of commercially derived fish, as well as the limited number and value of available broodstock, a non-lethal means of assessing individual reproductive status was necessary. Although there are several techniques used to assess maturity, such as ultrasound (Reimers et al., 1993; Blythe et al., 1994a,b), or the determination of plasma parameters (vitellogenins, progestins etc.), they can be costly, time consuming, or inappropriate.

An effective, yet cost efficient method for determining ovarian maturity in female fish is based on ovarian biopsies, whereby intra-ovarian oocytes are extruded from live fish by means of a glass or polyethylene cannula inserted via the oviduct. Usually this technique is employed

to either determine the sex of fish which display no external sexual dimorphic characteristics, to assess the maturational status prior to hormone treatment, or to predict spawning time after hormone treatment. The objectives of this study were to validate the use of cannulation for yellowtail flounder, to establish the effects of preservation on yellowtail flounder oocytes, and to use the cannulation procedure to follow the seasonal growth of yellowtail flounder oocytes.

3.2 Methods and Materials

Eight maturing females (252-589g; 29.0-35.0cm) were caught by SCUBA from Conception Bay, Newfoundland in November 1994 and held in 2000L tanks under ambient water temperatures and photoperiod. During the experimental period (March, 1995 - May, 1996), fish were placed in a 400L square tank, with three mature males. Ovarian biopsies were performed monthly, except during the group's spawning season (Jun-Aug). On several random occasions, cannula insertion was not possible in some fish. To prevent injury to these fish, sampling was not attempted if the cannula could not easily and rapidly be inserted into the ovary via the oviduct.

To determine whether oocytes removed by cannulation from the anterior ⅓ were characteristic of the entire ovary, right ovaries of five females were removed and divided into three equal length portions representing the anterior, middle, and posterior. Diameters of one hundred oocytes from each region were determined and pooled to represent the maturation state of the entire ovary.

3.3 Results

When the diameters of oocytes from the anterior ⅓ of the ovary (the sampling area of the cannula) were compared with oocyte diameters from the entire ovary, no difference was observed ($F = 0.3$, $P = 0.59$, $n = 2000$). Also, the diameters of oocytes that were removed by cannulation from the anterior ⅓ of the right ovary did not differ from oocytes removed from the same ovary after excision (two-tailed t-test; $t = 1.97$, $P = 0.83$, $n = 300$). When oocyte diameters were compared between the anterior ⅓ of both the left and right ovaries no difference was observed ($F = 0.15$, $P = 0.70$, $n = 300$).

Oocytes placed in a preservative solution of 1% formalin, 0.6% saline increased in volume after immersion (Fig. 3.1). Diameters of 20 oocytes were determined within 2 minutes. Initially, there is a rapid increase in volume which reached a maximum of 12.3 ± 4.5% ($\bar{x}$ ±SD, $n = 100$) within 24hr. After two weeks the volume had decreased to 9.4 ± 3.4%, and after one month to 8.1 ± 2.1%, i.e. greater than the initial measurement.

Spawning of this group of yellowtail flounder occurred between June and early August in 1995 and 1996 (Fig. 3.2). In September and October, after spawning, no oocytes could be removed by cannulation, however vitellogenic oocytes were first extracted in December and averaged 303 ± 18μm in diameter. Vitellogenesis continued until spawning with a significant difference in oocyte growth ($F = 257.8$, $P = 0.0001$, $n = 1592$) between each consecutive month of sampling. The maximum mean diameter attained for prespawning oocytes was 433 ± 39μm ($\bar{x}$ ±SD, $n = 399$)(Fig. 3.3). Since sample sizes differed between each monthly cannulation ($n = 224$-399), values are expressed as percent frequency. A minimum sample

size (Sokal and Rohlf, 1996) of 114 oocytes must be used in order to detect a 5% difference between two means at $\alpha = 0.05$.

3.4 Discussion

According to Harvey and Hoar (1979), successful implementation of the ovarian cannulation technique in teleosts must meet the following criteria: 1) the anatomy of the oviduct must permit direct passage of the cannula into the lumen of the ovaries, 2) since consistent positioning of the cannula within the ovary cannot always be attained, oocytes must be uniformly distributed throughout the ovary in order to provide a representative sample, and 3) diameters of oocytes must be known for all stages of maturity in order to determine whether mean diameter may be reliably used as an indicator of maturity.

The oviduct of the yellowtail flounder is short and empties into the cloaca slightly posterior and to the right of anus. The ovaries lie in ovarian cavities between the haemal spines and the body wall musculature, protruding slightly into the abdominal cavity and extending to the caudal peduncle (Howell, 1983)(Fig. 2.2). Location of the cannula within the ovaries can be determined by a slight pressure on the body surface above the ovaries. Because there was no difference in oocyte diameters between the left and right ovaries, all oocytes were removed from the anterior ⅓ of the right ovary.

All oocytes were removed from the anterior ⅓ of the right ovary and are representative of the maturity stage of oocytes throughout the entire ovary. Shehadeh et al. (1973) removed oocytes from seven distinct regions of grey mullet (*Mugil cephalus*) ovaries and noted that

oocytes from anywhere within the ovary, except the extreme anterior or posterior regions would provide a representative sample. In the present study, the extreme anterior and posterior regions of yellowtail flounder ovaries were not sampled.

Oocytes were placed in a preservative of 1% formalin in 0.6% saline solution to facilitate measuring and storage, and their diameters were investigated over short and relatively long periods of time. A rapid increase in oocyte diameter occurred initially, which reached a maximum of 12.3% volume after 24 hours. After a period of two weeks, the diameters of preserved oocytes decreased from the peak, and after one month were still 8.1% greater in size than the initial diameter (Fig. 3.1). Shehadeh et al. (1973) noted no change in oocyte volume for grey mullet oocytes placed in the same preservative, whereas Garcia (1989a) used a 5% phosphate buffered formalin solution to preserve sea bass (*Lates calcarifer*) oocytes and found no significant change in volume if diameters were determined within 1hr.

The potential effects of some preservatives on specific tissues have been shown to be dependant on several factors such as prefixation handling, type and strength of preservative, exposure time, and the size of the specimen being preserved (Heming and Preston, 1981; Tucker and Chester, 1984). An optimum preservative must be established individually for each species in order to standardize methods and reduce errors associated with swelling, shrinking or distortion (Takizawa et al., 1994). Heming and Preston (1981) noted a 16% increase within two days in the yolk sac weight of preserved chinook salmon alevins (*Oncorhynchus tshawytscha*). After the initial rapid increase, there was a decline in weight which stabilized after 12 days at 9.5% greater than the initial live measurement. Since

swelling of yellowtail flounder oocytes is initially rapid and reaches a peak around 24 hr., this represents the most appropriate time for determining oocyte diameters if rapid results are required; otherwise, diameters should be determined after volume changes have stabilized (c. >4wks).

The technique of ovarian cannulation has commonly been used for assessing the maturity of fish, especially immediately prior to the spawning season when hormone treatment is being administered. To our knowledge, only one other study exists (Kuo et al., 1974) in which ovarian cannulation was used solely for assessing ovarian maturity throughout the entire reproductive cycle of milkfish (*Chanos chanos*). Previous studies on yellowtail flounder have documented the annual reproductive cycle (Scott, 1947; Howell, 1983) and the batch fecundity and spawning frequency (Zamarro, 1991) of New England and Grand Banks yellowtail flounder, respectively. Spawning of Grand Banks fish occurs between May and early August, after which ovaries become flaccid and bloodshot possessing mainly previtellogenic oocytes and postovulatory follicles. Sampling by ovarian cannulation in post-spawned females (Aug.- Sept.) does not permit extrusion of the tightly clustered previtellogenic oocytes destined for recruitment into vitellogenesis.

Vitellogenic oocytes, opaque due to the incorporation of yolk, began development in early November (Linehan, 1996), which corresponds to an increase in plasma estradiol-17ß levels (Clearwater, 1997). Thereafter vitellogenic oocytes are easily removed, along with small clusters of previtellogenic oocytes. Clear previtellogenic oocytes range in size between 22-197μm, whereas opaque oocytes at the onset of vitellogenesis range in size between 175-

351μm; a range comparing favourably with Howell's (1983) 200μm vitellogenic oocytes. Vitellogenesis continues throughout the winter months, with oocytes steadily increasing in size until spawning (Fig. 3.2). The maximum diameter observed for a vitellogenic yellowtail flounder oocyte was 585μm, however the average maximum pre-spawning diameter was 433 ± 39μm ($\bar{x} \pm SD$, n = 400).

Yellowtail flounder oocytes progressively incorporate yolk and increase in diameter until final maturation and hydration. Similarly, winter flounder oocytes undergo a prolonged period of slowed growth beginning four to five months before spawning (Harmin et al., 1995). During this period, the upper range of oocyte diameters remain relatively unchanged while the smaller oocytes increase in size. Yellowtail flounder oocyte size distributions reach a maximum mode of approximately 400μm, beginning around early March (Fig. 3.3). Later in the season, a small percentage of oocytes attain diameters greater than 400μm, while the smaller oocytes decrease in abundance.

In summary, ovarian biopsies obtained from live fish using cannulation are an effective means for determining the *in vivo* status of oocyte maturation in yellowtail flounder. This basic assumption is based on a uniform oocyte distribution throughout both ovaries. Cannulation is effective for sampling maturing oocytes throughout the reproductive cycle, except in post-spawned females, and this information can be applied to determine when fish are mature enough to respond to hormone treatment when attempting to advance spawning.

Figure 3.1 Effects of preservative solution (1% formalin, 0.6% saline) on the percent volume change of oocytes over time (Log_{10} minutes). Vertical bars = ±SD. (n = 100 per sample)

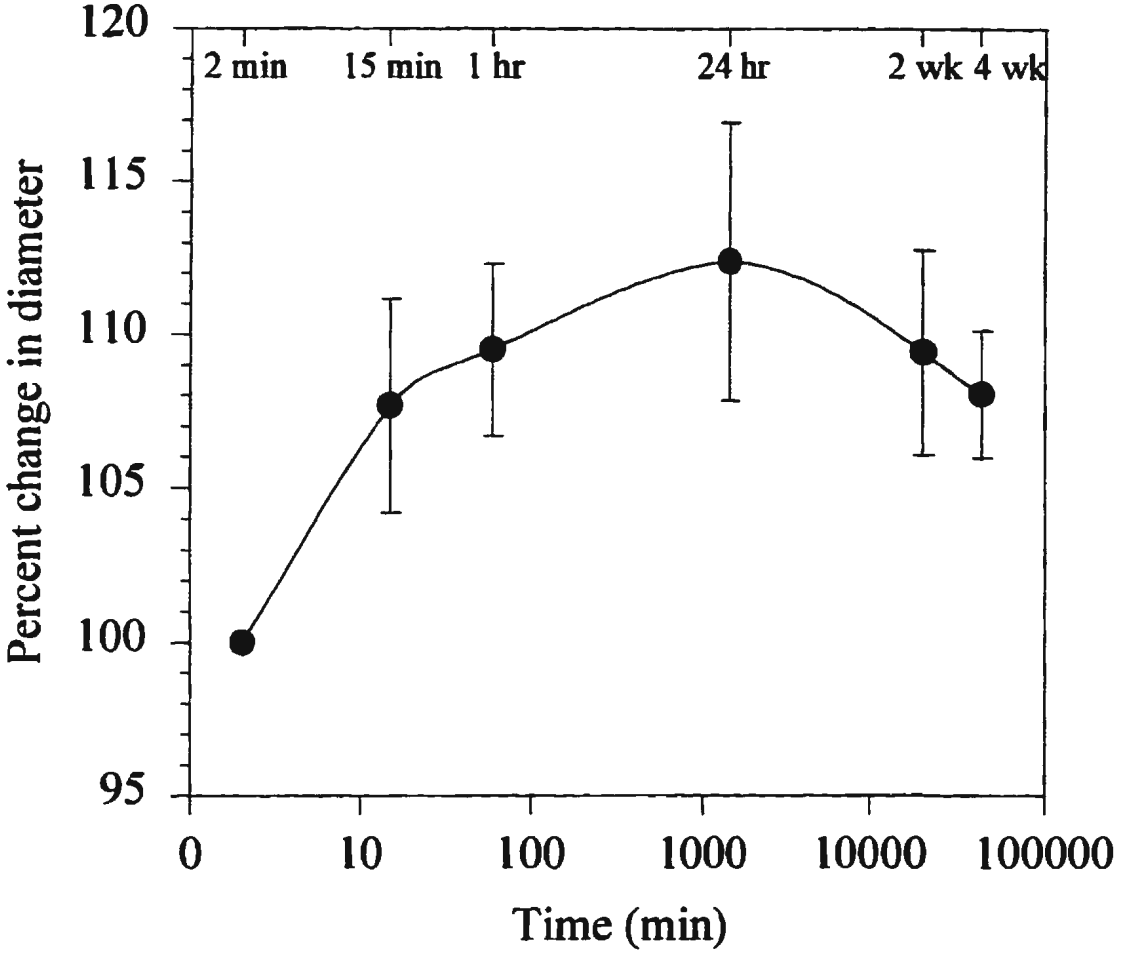

120
115
110
105
100
95
2 min
15 min
1 hr
24 hr
2 wk
4 wk
Percent change in diameter
0
10
100
1000
10000
100000
Time (min)

Figure 3.2 Annual vitellogenic oocyte growth (μm) in yellowtail flounder. Each point represents mean oocyte diameter for all fish (n = 6-8 fish). * represent cannulation sampling from which no oocytes were obtained. Vertical bars = ±SD

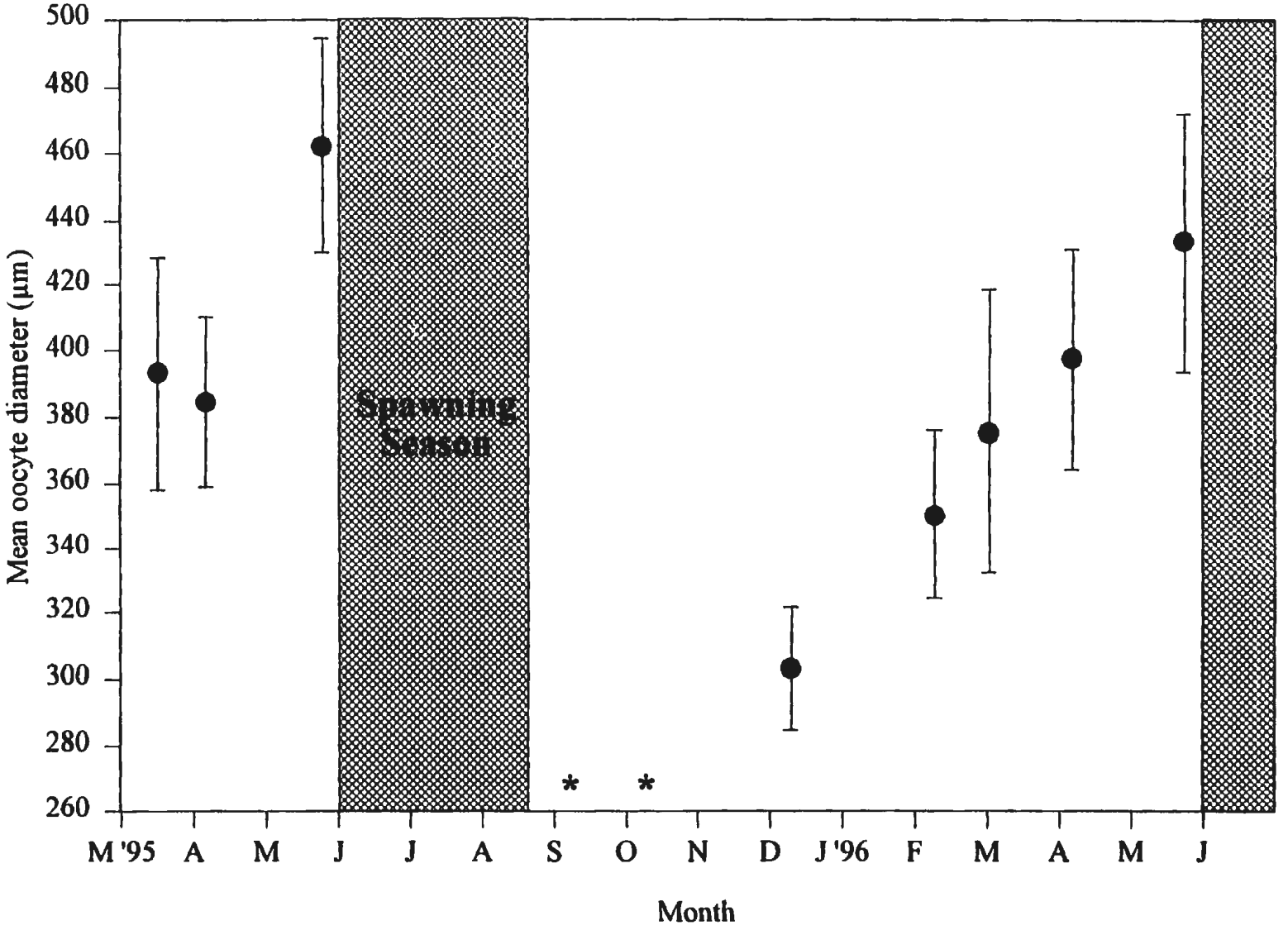
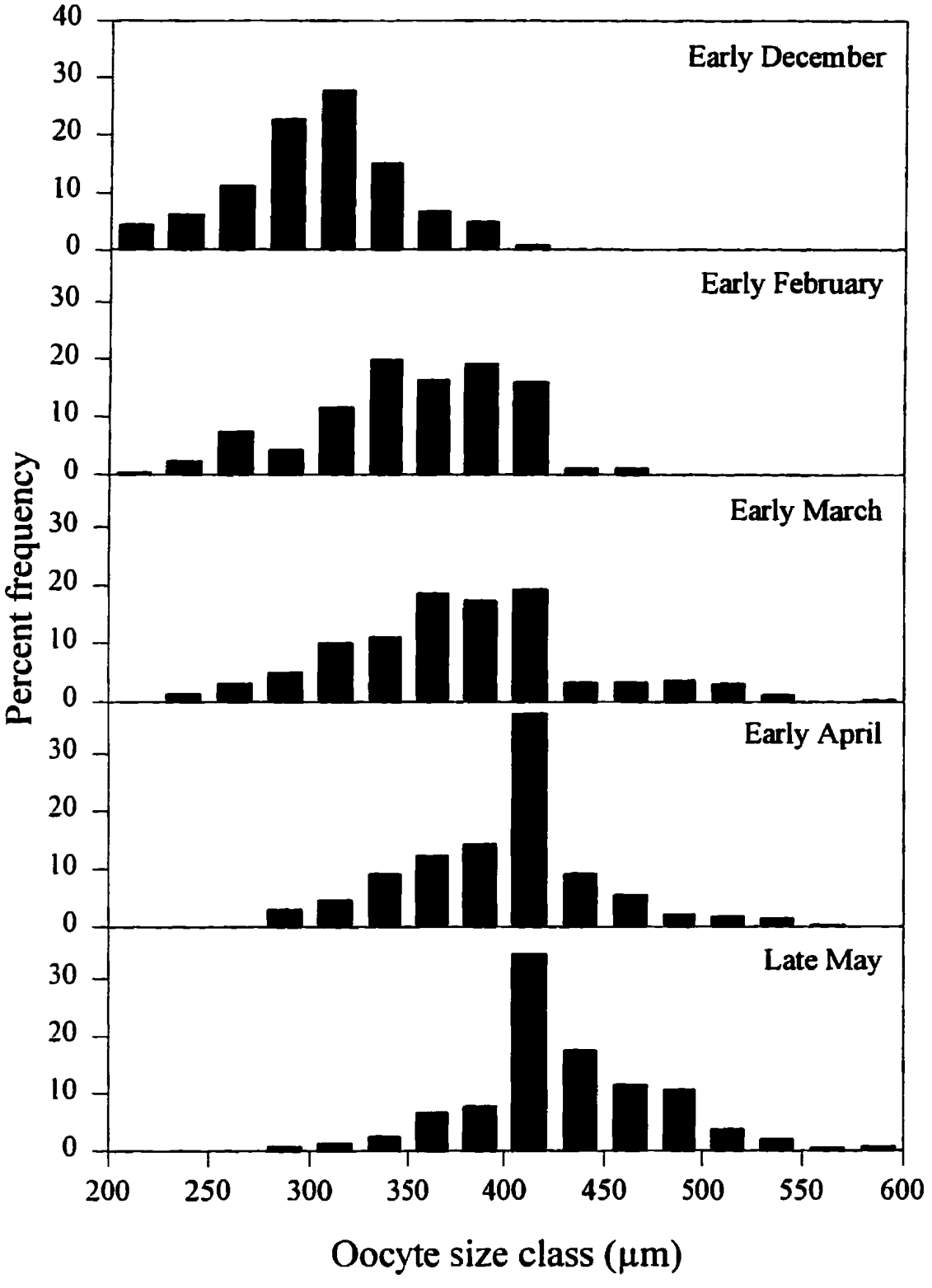

500
480
460
440
420
400
380
360
340
320
300
280
260
Mean oocyte diameter (µm)
Spawning Season
*
*
M '95
A
M
J
J
A
S
O
N
D
J '96
F
M
A
M
J
Month

Figure 3.3 Percent frequency of vitellogenic oocyte size classes (μm) pooled from fish sampled at consecutive months throughout the reproductive cycle.


Early December
Early February
Early March
Early April
Late May
Percent frequency
0
10
20
30
40
200
250
300
350
400
450
500
550
600
Oocyte size class (μm)

Chapter 4

Advanced ovulation in yellowtail flounder using gonadotropic hormone-releasing hormone analogue (GnRHa)

4.1 Introduction

The yellowtail flounder (*Pleuronectes ferrugineus* Storer 1839) is a commercially important flatfish species distributed throughout the Northwest Atlantic, with peak recorded landings of 39,000t in 1972 (Walsh et al., 1995). With a dramatic decline in all five of the major Northwest Atlantic yellowtail flounder stocks, interest in developing aquaculture of this species is increasing. Despite a large literature base which exists relating to fisheries data, little is known about many basic and fundamental aspects of yellowtail flounder biology. There have been several studies on the reproduction of yellowtail flounder (Scott, 1947; Howell, 1983; Zamarro, 1991). Yellowtail flounder conform to the description of annual determinate group-synchronous spawners (Wallace and Selman, 1981) that produce an average of 14-22 serial egg batches, usually daily, throughout the spawning season (Manning, 1997). Spawning usually occurs from April to June in the southern limits of their range, Chesapeake Bay (Bigelow and Schroeder, 1953), whereas at the northern limits of their range, the Grand Banks, the spawning season occurs between June and August (Pitt, 1970).

Under captive conditions, many fish may have difficulty reproducing normally, presumably due to a lack of appropriate environmental or hormonal cues, and thus they may fail to ovulate or spontaneously spawn (Bye, 1990). Induction of spawning is often possible

with the use of gonadotropic hormone-releasing hormone analogues (GnRHa), which are gaining widespread acceptance over the classical steroid or pituitary hormone treatments because of their ease of use, small required doses, and nonimmunogenic characteristics (Zohar, 1989; Crim and Bettles, 1997). Captive yellowtail flounder undergo gonadal recrudescence, including the reproductive processes of vitellogenesis, final egg maturation and ovulation. They do not spawn and release eggs when held in shallow tanks, and must therefore be manually stripped on a daily basis. Such repetitive handling can be stressful and may have detrimental effects on gamete and larval quality (Campbell et al., 1992; Campbell et al. 1994; Short et al., 1996). Using GnRHa, Larsson et al. (1997) synchronized ovulation in groups of yellowtail flounder, at the beginning of the natural spawning season. Egg quality, measured by egg fertilization and hatch rates, was retained or even improved in GnRHa treated females compared with controls.

Few studies have attempted to maximize the seasonal advancement of spawning using GnRHa (Sanborn and Misitano, 1991; Harmin et al., 1995). Advancing spawning can extend the spawning season and improve overall hatchery productivity. The first objective of this study was to advance spawning of yellowtail flounder, and determine if a minimum oocyte diameter must be attained for hormonal induction of ovulation. The second objective was to use sustained administration of GnRHa to advance ovulation, and determine if advancing ovulations would have detrimental effects on egg quality.

4.2 Materials and Methods - Experimental Design

Prior to the natural spawning season for yellowtail flounder, between June and August, three separate GnRHa trials were conducted with maturing females beginning either 1) April 1995, 2) June 1995, or 3) February 1996. During the first two trials, groups of eight females were implanted with GnRHa either on 1) April 21 or 2) June 1, 1995, approximately two months and two weeks prior to the expected spawning season, respectively. Another group of eight females, receiving blank cholesterol pellet implants on April 21, served as a control for both of these trials. Following treatment, the females were checked for ovulation and stripped on a daily basis once ovulating. The volume and the quality of all individual egg batches collected were determined throughout the spawning season.

On February 29, 1996, four months prior to the expected spawning season, the third spawning trial was conducted comparing two separate groups of eight females, which were either implanted with blank cholesterol pellets (control) or pellets containing GnRHa. Again, females were checked for ovulation and stripped daily once ovulating. However, the quantity and quality of individual egg batches were determined only for females in the hormone-treated group.

4.3 Results

Ovulations were observed in some females from all of the GnRHa treated groups, however the best response occurred in fish treated the closest to the natural spawning season. Females implanted in February had mean initial oocyte diameters ranging from 232μm to

396μm (Table 4.1). Only two of eight females ovulated and they had the largest oocyte diameters. All females implanted in April and June ovulated, and possessed mean oocyte diameters ranging between 399-512μm and 411-516μm, respectively (Table 4.1).

Initial oocyte diameters were compared with spawning response, and a significant relationship was observed in GnRHa treated fish (ANOVA; $F = 36.22$, $P < 0.0001$, $n = 22$). The mean oocyte diameter for all spawning GnRHa treated fish ($n = 16$) was 447μm, whereas the mean oocyte diameter for nonspawning fish ($n = 6$) was 316μm. There was no relationship between spawning performance and oocyte diameters in control females [1995], with spawning fish ($n = 5$) having a mean oocyte diameter of 446μm, and nonspawning fish ($n = 3$) having a mean oocyte diameter of 401μm (ANOVA; $F = 4.06$, $P = 0.09$, $n = 8$).

Closer to the natural spawning season (June), ovulatory responses became more uniform and synchronized. For example, time to ovulation for the two spawning fish implanted in February were 18 and 32 days (Fig. 4.1). The mean time to ovulation for GnRHa treated females implanted in April was 20 days (Table 4.1) and 86% of the females were ovulating within 6 days of the first ovulating female (Fig 4.1). For females implanted with GnRHa in June, the mean time to ovulation was 14 days (Table 4.1) and all females were ovulating within 5 days of the first ovulating female (Fig 4.1). No relationship was observed between initial oocyte diameter and time to ovulation. Since one fish died between implantation and the first group ovulation in April and June, cumulative % ovulated (Fig. 4.1) refers to the percentage of fish surviving to at least the first ovulation.

Egg volumes from all stripped fish were pooled to represent a daily group spawning profile (Fig. 4.2). The group of fish implanted in June had the shortest overall spawning duration of 40 days, yet produced the largest number (total volume) of eggs during their spawning period (Table 4.2). Also, this group produced larger mean egg volumes, a greater mean number of batches per fish, and a greater mean egg volume per batch although there were no significant differences between each treatment (Table 4.2).

Indicators of egg quality were studied in each trial, using egg viability rates, egg fertilization rates, and hatch rates (Fig. 4.3). Mean egg viability rates, which were high for all groups, were either equal or marginally higher for eggs produced by GnRHa treated fish compared with control fish. For egg fertilization and hatch rates, the mean egg fertilization rates produced by all GnRHa treatments were greater than the control group, and hatch rates were either equal or marginally higher than the control group. Given all three parameters, the best egg quality was produced by females implanted with GnRHa in June. The effect of individual batches explained a large percentage of the variance for each of the egg quality parameters. For viability rates, 61% of the variance was due to the egg batch effect, 28% was due to differences between fish, whereas 8% was due to the treatment effect (total model r^2 = 0.97). Similarly for fertilization rates, batches accounted for 66%, fish for 21% and treatment 9% of the variance (total model $r^2 = 0.96$). However for hatch rates, the variances due to treatment and fish were only 3% and 13%, respectively. Most of the variance (72%) was attributable to batch effect (total model $r^2 = 0.88$).

4.4 Discussion

The results of the present study showed that GnRHa advances spawning in female yellowtail flounder without any apparent detrimental effects on egg quality. This is important because a broadening of the spawning season leads to more efficient management of a broodstock facility and may provide year-round production (Lam, 1983; Zohar, 1989). In this study, it was demonstrated that the ovulation in yellowtail flounder can be advanced by nearly 3 months (Fig. 4.1). However, the results were not optimized using GnRHa implantation in February, since only 25% of the females responded. Later in the season, the GnRHa treated females ovulated more predictably, suggesting that prespawning fish are most responsive close to the natural spawning season. Indeed, the shortest time to ovulation occurred after GnRHa treatment of female yellowtail flounder in June. Similar results have been found with other species where spawning was advanced with GnRHa treatment of Atlantic salmon, *Salmo salar* (Crim and Glebe, 1984; Taranger et al., 1992), coho salmon, *Oncorhynchus kisutch* (Fitzpatrick et al., 1984), sockeye salmon, *O. nerka* (Slater et al., 1995) and winter flounder, *Pleuronectes americanus* (Harmin and Crim, 1992).

Vitellogenesis in yellowtail flounder begins between October and November, when oocytes are approximately 178μm in diameter. Vitellogenesis, or the production and sequestration of vitellogenin into the oocytes, increases both the opacity and diameters of the oocytes, and continues until the spawning season in June (CHAPTER 3). It has been suggested that oocytes must grow to a minimum critical size before they can be induced to undergo final maturation by hormone treatment (Garcia, 1989b). The mean initial oocyte diameter for all

ovulating yellowtail flounder, in both control and the GnHRa treatment groups was 446μm and 447μm, respectively. Each ovulating fish treated with GnRHa had a mean initial oocyte diameter greater than 396μm. However, one ovulating fish treated in February had a mean initial oocyte diameter of 354μm, and seems anomalous since several other fish possessed similar sized oocytes yet did not ovulate. In yellowtail flounder, it is thus suggested that GnRHa may be used to induce ovulation if the mean oocyte diameter is at least 400μm. Some studies have demonstrated a relationship between increased oocyte size and a shortening of the time to ovulation (Tamaru et al., 1988; Garcia, 1989b; Larsson et al., 1997), but no such relationship was observed in this study.

Egg quality is defined as the potential of an egg to produce viable fry. Fertilization rates, hatch rates, and occasionally larval survival to first feeding have all been used as indicators of egg quality (Kjørsvik et al., 1990). Determination of egg viability rates, or the percentage of potentially fertilizable eggs, is an attempt to develop a practical egg quality parameter which is solely based on the morphological characteristics of unfertilized egg for yellowtail flounder, i.e. translucent, floating, spherical, and undimpled eggs lacking a perivitelline space (Larsson et al., 1997). Although this procedure is relatively simple, when compared to determining fertilization rates, the use of viability rates to determine egg quality is subjective and may overestimate the eggs potential to be fertilized, since we found not all 'viable' eggs become fertilized.

Egg quality for GnRHa treated fish, based on fertilization rates and hatch rates was not reduced, and in some cases was enhanced. Fertilization rates for all GnRHa treated groups

of females were higher than the control group which had an egg fertilization rate of 54.6%. The fertilization rates for the control group compare favourably to those for untreated yellowtail flounder in other studies (Larsson et al., 1997; Manning, 1997). Fitzpatrick et al. (1984) reported no decrease in egg fertilization rates in coho salmon treated with LHRHa induced to spawn one month ahead of the natural spawning season. Similarly, no significant effects of hormone treatment were observed in sea bass (Garcia, 1989c). Hatch rates obtained from fish in the four month group (February) in the present study were equal to the control group, whereas both the two month group (April) and the two week group (June) were approximately 6% higher than the control. However, this small difference is insignificant on a practical scale. Larval quality also did not seem impaired and the percentage of curved larvae for all treatments ranged between 1.7 and 5.9%, whereas the percentage of dead larvae ranged between 0.1 and 0.5%. It appears that egg quality of yellowtail flounder is not diminished either by GnRHa treatment or by advancing spawning. If the fish is capable of undergoing the processes of final maturation, it seems to produce quality eggs similar or better than those of untreated fish.

There are many reports, however of decreased egg quality after hormone induction of spawning in fish, especially when induced to spawn in advance of their natural spawning season. Smigielski (1979) used carp pituitary extract to study induced spawning in yellowtail flounder three to four months ahead and during the natural spawning season. Advancement of ovulation did not occur because oocytes were previtellogenic, however, fertilization rates for eggs obtained by hormonal induction during the spawning season were described as

'satisfactory'. No comparisons could be made to control fish since they did not spawn. A lowering of fertilization rates was also observed in common sole (*Solea solea*) treated with high doses of human chorionic gonadotropin (Ramos, 1986b), or GnRHa (Ramos, 1986a), although the possible causes were not discussed. Crim and Glebe (1984) treated Atlantic salmon with GnRHa one month prior to natural spawning and concluded that poor egg quality was a consequence of incomplete ovulation or over-ripening, whereas Mylonas et al. (1992) attributed poor egg quality in brown trout, treated with GnRHa 16 days before natural spawning, to premature ovulation before meiosis was completed.

Our findings indicate that the use of GnRHa is an effective means for advancing and/or synchronizing spawning of yellowtail flounder, even if applied seven weeks prior to the natural spawning season. Perhaps the spawning season could be further advanced, with more synchronous results, if GnRHa treatment was combined with other forms of reproductive manipulation, such as photoperiod or temperature adjustments. However, no acceleration of vitellogenesis was observed in yellowtail flounder by photoperiod manipulation and it was concluded by Linehan (1996) that photo-adjustments must be established before the onset of vitellogenesis. Similar photoperiod manipulation of flatfish, such as the dab, *Pleuronectes limanda* (Scott et al., 1980), turbot, *Scophthalmus maximus* (Devauchelle et al., 1988), and sole, *Solea solea* (Devauchelle et al., 1987) has resulted in advanced ovulation, but often the individuals are poorly synchronized.

Gamete quality was not reduced in yellowtial flounder as seen in some other studies, but rather in some cases enhanced by the use of GnRHa. Perhaps there is a biological limit as to

how far ahead of the natural spawning season yellowtail flounder can be advanced to spawn using GnRHa. While it is recognized there are many complex hormonal interactions preceding spawning, a useful indicator for receptiveness to hormone treatment is oocyte size. It is suggested that yellowtail flounder oocytes should be approximately 400μm before they can be induced to enter the processes of final maturation and ovulation.

The dosage of GnRHa used in this study (100μg/pellet; ≈200μg/kg fish BW) was sufficient to induce ovulations in most cases, however, for economic reasons further studies should determine the minimum dosages required to elicit a spawning response in yellowtail flounder at various times throughout the reproductive cycle. Many forms of GnRH analogues, each with varying potencies are commercially available - the most commonly used forms being [D-Ala^6, Pro^9 NEt]LHRHa and [D-Arg^6, Pro^9 Net]sGnRH. Several analogues should be tested to determine the most effective. Since cholesterol pellets are not biodegradable, perhaps a more appropriate vehicle of administration should also be considered.

Future studies on the reproduction of yellowtail flounder ought to address the combination of GnRHa treatment and other forms of reproductive manipulation such as photoperiod and temperature. In addition, since captive yellowtail flounder do not spontaneously release gametes when held in shallow tanks, attempts should be made to define the conditions for spawning and eliminating daily handling, leading to improved broodstock management protocols.

Table 4.1 Average initial oocyte diameter (μm) and time to ovulation (d) for individual fish in each treatment group.

Implant date	Fish ID	Oocyte Diameter mean ± s.d. (μm)	Time to Ovulation (d)	Implant date	Fish ID	Oocyte Diameter mean ± s.d. (μm)	Time to Ovulation (d)
February 1996	6G	232 ± 19	DNO	**April 1995**	2O	399 ± 31	35
	6P	299 ± 21	DNO		2R	417 ± 32	17
	6Y	332 ± 40	DNO		2B	424 ± 30	15
	6O	339 ± 39	DNO		2V	427 ± 18	DIED
	6V	344 ± 25	DNO		2P	449 ± 39	21
	6R	353 ± 35	DNO		2W	456 ± 20	20
	6W	354 ± 39	32		2Y	474 ± 25	20
	6B	396 ± 56	18		2G	512 ± 22	15
June 1995	4V	411 ± 30	17	**Control 1995**	3R	370 ± 24	DNO
	4O	424 ± 19	DIED		3Y	383 ± 25	DNO
	4P	444 ± 25	13		3O	424 ± 32	68
	4B	445 ± 28	15		3V	429 ± 31	63
	4W	476 ± 31	16		3P	439 ± 31	53
	4Y	489 ± 29	13		3W	450 ± 23	DNO
	4G	496 ± 23	13		3G	464 ± 28	54
	4R	516 ± 28	12		3B	474 ± 27	58
				Control 1996 *			

DNO - Did not ovulate.

* - Control group 1996. Eight females in group. None spawned before June 1996, when three females began spawning during the second week.

Figure 4.1 Cumulative percent ovulated for fish implanted with GnRHa four months (●), two months (■), and two weeks (▲) prior to the normal spawning season. Days before natural spawning calculated with reference to controls (▼). Arrows indicate day of implantation.

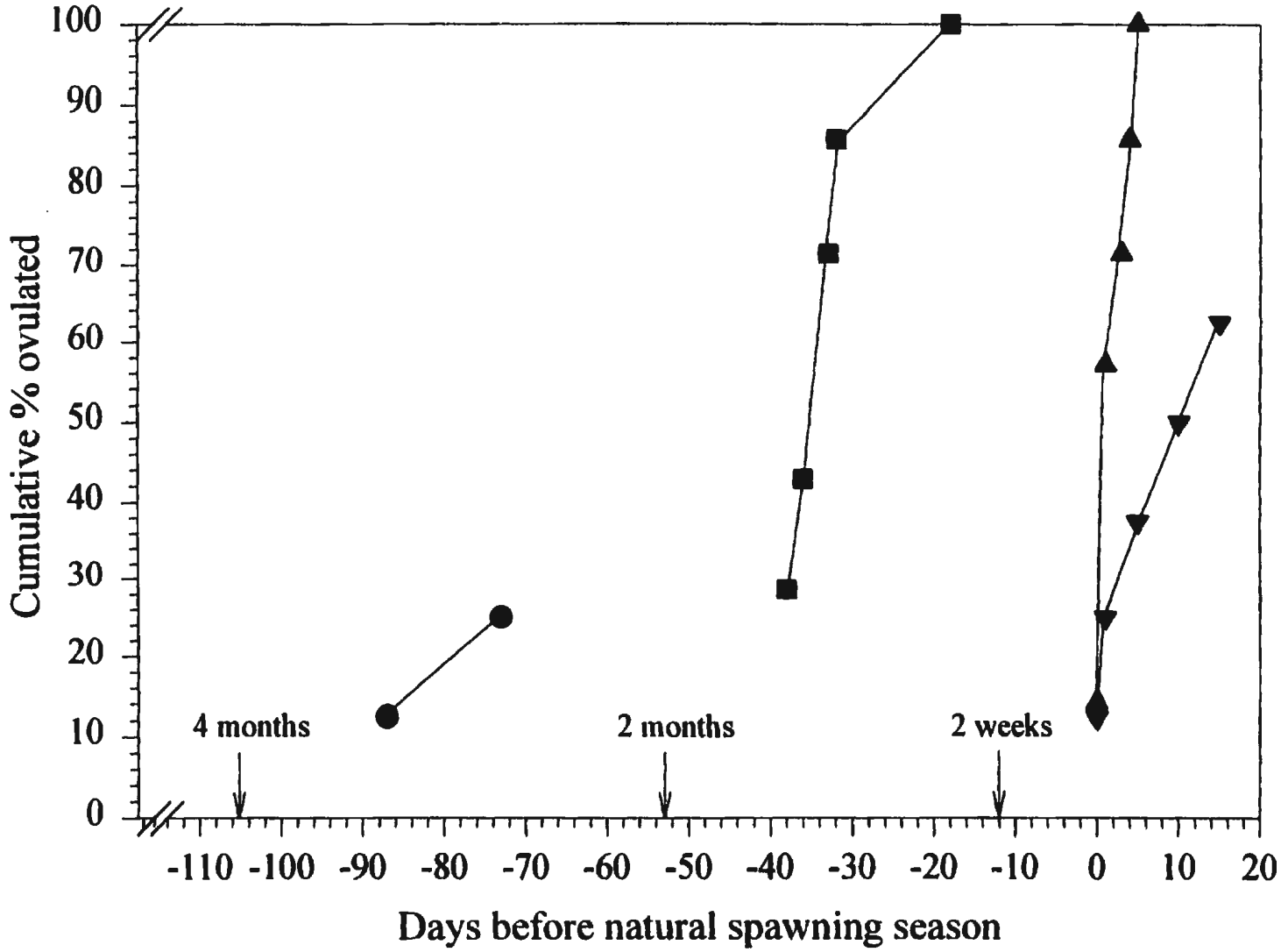

Cumulative % ovulated
100
90
80
70
60
50
40
30
20
10
0
4 months
2 months
2 weeks
-110 -100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20
Days before natural spawning season

Figure 4.2 Daily egg volumes (mL), pooled from all ovulating females.

a) females implanted four months ahead of the normal spawning season (n = 2)

b) females implanted two months ahead of the normal spawning season (n = 7)

c) females implanted two weeks ahead of the normal spawning season (n = 7)

d) naturally ovulating control females (n = 5)

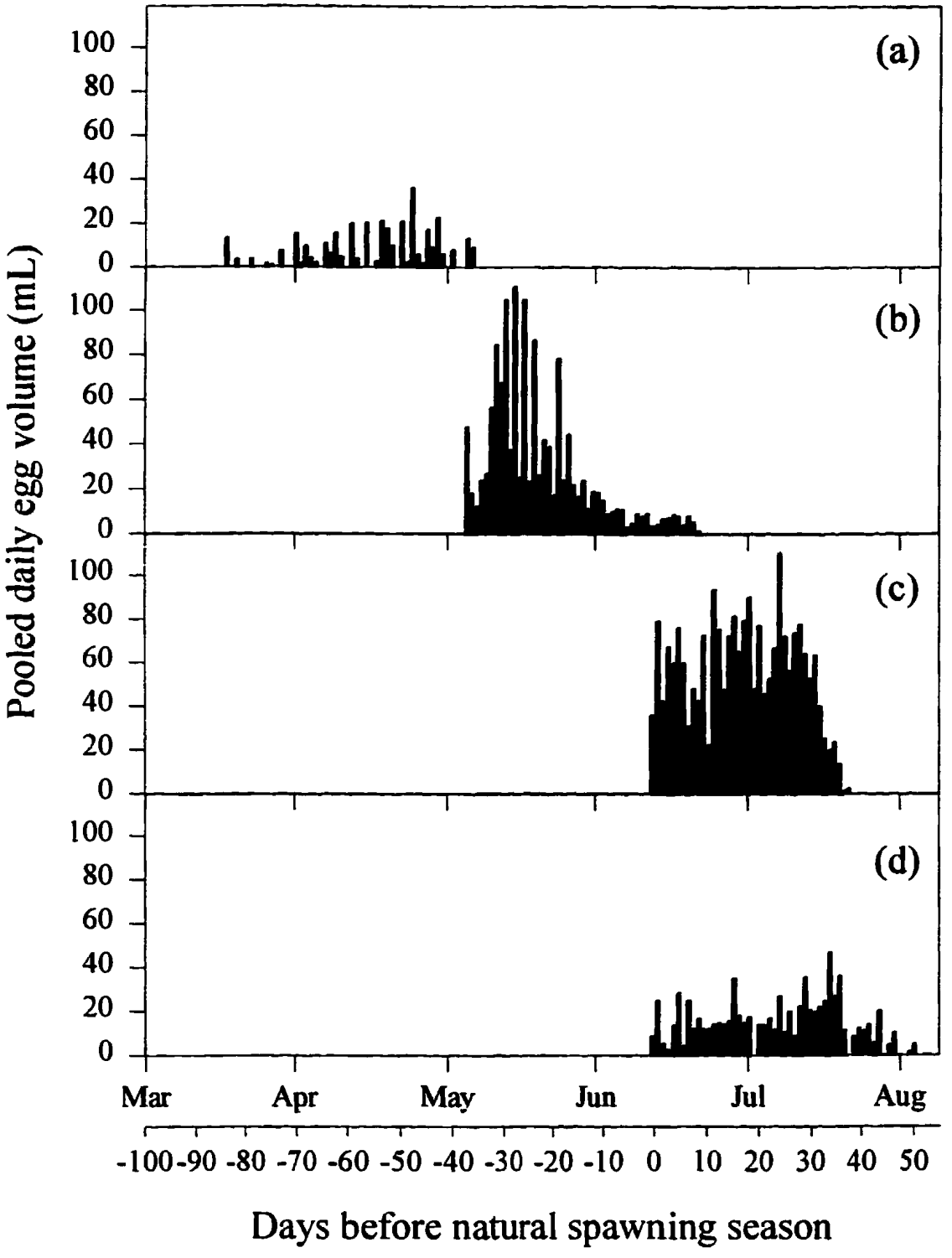

Pooled daily egg volume (mL)
(a)
(b)
(c)
(d)
100
80
60
40
20
0
Mar
Apr
May
Jun
Jul
Aug
-100 -90 -80 -70 -60 -50 -40 -30 -20 -10 0 10 20 30 40 50
Days before natural spawning season

Table 4.2 Comparison of spawning results from each GnRHa treated group with naturally spawning fish (Control).

	GnRHa implantation			
	February 29 (n = 2)	April 21 (n = 7)	June 1 (n = 7)	Control (n = 5)
Duration of group spawning period (d)	51	48	40	53
Mean ± SD duration of individual spawning (d) [a]	43 ± 11	28 ± 12	23 ± 13	34 ± 17
Total egg volume (mL) *	359	1364	2220	757
Mean ± SD individual egg volume (mL) [b]	179.5 ± 108.9	194.9 ± 65.0	318.5 ± 256.6	151.4 ± 134.3
Mean ± SD individual number of egg batches [c]	16.5 ± 2.1	16 ± 5.2	18.9 ± 10.7	15 ± 13.4
Mean ± SD egg volume per batch (mL) [d]	9.3 ± 4.7	12.3 ± 4.3	15.7 ± 7.1	10.8 ± 2.8

* including volumes <3mL

a ANOVA; F = 1.42, $P = 0.27$, n = 21

b ANOVA; F = 1.11, $P = 0.37$, n = 21

c ANOVA; F = 0.18, $P = 0.91$, n = 21

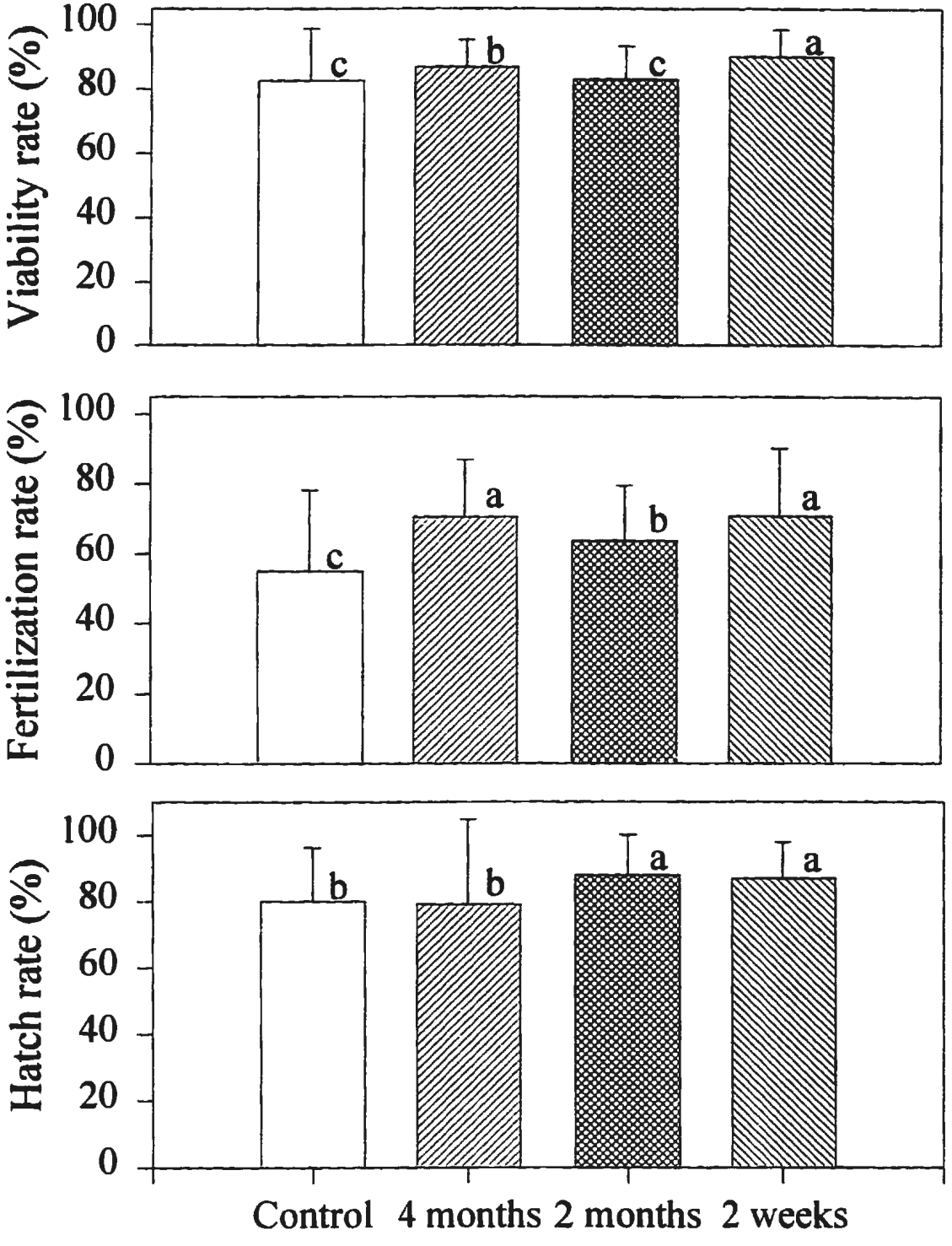
d ANOVA; F = 1.30, $P = 0.31$, n = 21

Figure 4.3 Egg quality indicators for GnRHa treated females compared with naturally spawning control fish. a) mean ±SD percent egg viability, b) mean ±SD percent egg fertilization, and c) mean ±SD percent hatch rates for the three GnRHa trials compared to the control group. Vertical bars indicate ±SD. Bars with same letters are not significantly different.

a) $F = 55.91$, $P = 0.0001$, $n = 1052$

b) $F = 49.43$, $P = 0.0001$, $n = 1010$

c) $F = 13.96$, $P = 0.0001$, $n = 1002$


Viability rate (%)
100
80
60
40
20
0
c
b
c
a
Fertilization rate (%)
100
80
60
40
20
0
c
a
b
a
Hatch rate (%)
100
80
60
40
20
0
b
b
a
a
Control
4 months
2 months
2 weeks

Chapter 5

Conclusions

Yellowtail flounder was chosen as a potential flatfish species for cold-ocean aquacultural development in Newfoundland in 1992. Previous flatfish studies had focussed on the Atlantic halibut, but the life history of this species was naturally demanding and progress has been limited. Yellowtail flounder was selected over several other indigenous flatfish species based on many criteria, including relatively simple life history traits (Goff, 1993; Crim, 1993). Although yellowtail flounder had been kept under captive conditions on several occasions, any attempt to rear them through an entire life cycle seems to be lacking.

Successful aquaculture of a new species depends partly on a thorough knowledge of the reproductive strategy of that species. Previous studies had addressed several aspects of yellowtail flounder reproduction including oogenesis and fecundity (Howell, 1983; Zamarro, 1991), respectively. It was the intent of this study to follow oocyte growth during the annual reproductive cycle of a group of captive fish by employing a non-lethal sampling technique - ovarian cannulation. This technique allows for repeat sampling of individuals, thus reducing intraspecific variation.

The natural spawning season occurs between June and August when individuals may spawn as many as 22 batches, before becoming 'spawned-out'. After spawning ceases, ovaries regress until October/November when vitellogenesis is resumed and vitellogenic oocytes, approximately 200μm in diameter, can be removed by cannulation. Vitellogenesis,

and thus oocyte growth, continues steadily until spawning, when oocytes reach a maximum mean diameter of 433μm (CHAPTER 3).

A previous study (Larsson et al., 1997) showed that yellowtail flounder respond well to GnRHa when the hormone is administered close to the natural spawning season. The time to first ovulation after treatment was brief and the spawning periods within the group were synchronized when compared to the control fish. Also egg quality, as determined by egg fertilization rates and hatch rates, was significantly enhanced. Based on this knowledge, it was the intent of this study to apply GnRHa treatment at several stages throughout the reproductive cycle to promote ovulation, and to use oocyte diameter as a predictor for reproductive maturity.

Four months ahead of the natural spawning season (three months after the onset of vitellogenesis), mean oocyte diameters were 331μm. Eighteen and 32 days after GnRHa treatment, only two of eight fish responded and both females had the largest initial oocyte diameters of the group. Egg quality was similar or better than that attained for naturally spawning fish. It is suggested that once oocytes reach approximately 400μm in diameter, ovulation may be induced with GnRHa without any compromise to egg quality. Two months ahead of the natural spawning season, mean oocyte diameters were 445μm. By this time, all fish responded to GnRHa and were ovulating within 15-35 days after hormone treatment. Egg quality was similar or greater than that obtained from naturally spawned fish. Similarly, females implanted with GnRHa two weeks prior to the natural spawning season produced high quality eggs. However, the time to ovulation shortened to approximately two weeks,

egg batch volumes were increased, the number of egg batches was increased, and the duration of spawning was reduced (CHAPTER 4).

GnRHa can serve several purposes for controlling the spawning of captive broodstock. Spawning can be advanced without detrimental effects on egg quality, thus extending the spawning season, increasing hatchery productivity, and completing spawning before the summer rise in water temperatures, which would provide more suitable conditions for larval growth. In addition, GnRHa can synchronize the spawning of broodstock and shorten the duration of the spawning season, thus reducing handling stress and hatchery labour.

References

Ako, H., C.S. Tamaru and C.S. Lee. 1994. Chemical and physical differences in milkfish (*Chanos chanos*) eggs from natural and hormonally induced spawns. *Aquaculture* 127: 157-167.

Almendras, J.M., C. Duenas, J. Nacario, N.M. Sherwood and L.W. Crim. 1988. Sustained hormone release. III. Use of gonadotropin releasing hormone analogues to induce multiple spawnings in sea bass, *Lates calcarifer*. *Aquaculture* 74: 97-111.

Alvariño, J.M.R., M. Carrillo, S. Zanuy, F. Prat and E. Mañanos. 1992. Pattern of sea bass oocyte development after ovarian stimulation by LHRHa. *J. Fish Biol.* 41: 965-970.

Anonymous. 1932. First list of fishes in the Newfoundland fishing area. Annu. Rep. Newfoundland Fish Research Comm. for 1931. 1(4):107-110.

Bettles, S. 1996. Current status of yellowtail flounder (*Pleuronectes ferrugineus*) culture, and the potential for further commercial development. A report submitted to Canadian Centre for Fisheries Innovation, Memorial University of Newfoundland, St. John.s, Newfoundland. 13p.

Bigelow, H.B. and W.C. Schroeder. 1953. Fishes of the Gulf of Maine. *U.S. Fish and Wildl. Serv., Fish. Bull.* 53, 557p.

Blythe, B., L.A. Helfrich, W.E. Beal, B. Bosworth, and G.S. Libey. 1994a. Determination of sex and maturational status of striped bass (*Morone saxatilis*) using ultrasonic imaging. *Aquaculture* 125: 175-184.

Blythe, B., L.A. Helfrich, G.S. Libey, and W.E. Beal. 1994b. Induced maturation of striped bass, *Morone saxatilis*, exposed to 6, 9, and 12 month photothermal regimes. *J. World Aqua. Soc.* 25(2): 183-192.

Breton, B. L., Roelants, T. Mikolajczyk, T. Epler, and F. Ollevier. 1995. Induced spawning in teleost fish after oral administration of GnRH-A. p.102-104. *In*: P. Thomas and F.W. Goetz, (eds.). Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Austin, Texas.

Breton, B., C. Weil, E. Sambroni and Y. Zohar. 1990. Effects of acute versus sustained administration of GnRHa on GTH release and ovulation in the rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 91: 373-383.

Busch, R.L. and J.A. Steeby. 1990. An evaluation of a LHRH analog to induce spawning of channel catfish *Ictalurus punctatus*. *J. World Aqua. Soc.* 21: 10-15.

Bye, V.J. 1990. Temperate marine teleosts. p.125-143. *In*: A.D. Munro, A.P. Scott, and T.J. Lam (eds.). Reproductive Seasonality in Teleosts: Environmental Influences. CRC Press, Inc., Florida.

Campbell, P.M., T.G. Pottinger, and J.P. Sumpter. 1994. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. *Aquaculture* 120:151-169.

Campbell, P.M., T.G. Pottinger, and J.P. Sumpter. 1992. Stress reduces the quality of gametes produced by rainbow trout. *Biol. Reprod.* 47:1140-1150.

Carolsfeld, J., N.M. Sherwood, H. Kreiberg and S.A. Sower. 1988. Induced sexual maturation of herring using GnRH 'quick-release' cholesterol pellets. *Aquaculture* 70:169-181.

Clearwater, S.J. 1997. The reproductive physiology of yellowtail flounder (*Pleuronectes ferrugineus*) with an emphasis on sperm physiology. Ph.D. Thesis. Memorial University of Newfoundland, St. John's, Newfoundland. 195p.

Copeland, P.A. and P. Thomas. 1989. Control of gonadotropin release in the Atlantic croaker (*Micropogonias undulatus*): Evidence for lack of dopaminergic inhibition. *Gen. Comp. Endocrinol.* 74: 474-483.

Crim, L.W. and S. Bettles. 1997. The use of GnRH analogues in fish culture. In press. *In*: R. Nagabhushanam and M. Fingermann (eds.). Advance in Marine Biotechnology, Vol. 1. Oxford and IBH Publishing Company. New Delhi. In press.

Crim, L.W. 1993. Cold-water aquaculture of yellowtail flounder, *Pleuronectes ferrugineus*: pathways to commercial applications. A report submitted to Canadian Centre for Fisheries Innovation, Memorial University of Newfoundland, St. John.s, Newfoundland. 15p.

Crim, L.W., B.D. Glebe and A.P. Scott. 1986. The influence of LHRH analog on oocyte development and spawning in female Atlantic salmon, *Salmo salar*. *Aquaculture* 56: 139-150.

Crim, L.W. and B.D. Glebe. 1984. Advancement and synchrony of ovulation in Atlantic salmon with pelleted LHRH analogue. *Aquaculture* 43:47-56.

Crim, L.W., A.M. Sutterlin, D.M. Evans, and C. Weil. 1983. Accelerated ovulation by pelleted LHRH analogue treatment by spring-spawning rainbow trout (*Salmo giardneri*) held at low temperature. *Aquaculture* 35:299-307.

Devauchelle, N., J.C. Alexandre., N. Le Corre, and Y. Letty. 1988. Spawning of turbot (*Scopthalmus maximus*) in captivity. *Aquaculture*. 69: 159-184.

Devauchelle, N., J.C. Alexandre., N. Le Corre, and Y. Letty. 1987. Spawning of sole (*Solea solea*) in captivity. *Aquaculture*. 66: 125-147.

Drori, S., M. Ofir, B. Levavi-Sivan, and Z. Yaron. 1994. Spawning induction in common carp (*Cyprinus carpio*) using pituitary extract or GnRH superactive analogue combined with metoclopramide: analysis of hormone profile, progress of oocyte maturation and dependence on temperature. *Aquaculture*. 119:393-407.

Fermin, A.C. 1991. LHRH-a and domperidone-induced oocyte maturation and ovulation in bighead carp, *Aristichthys nobilis* (Richardson). *Aquaculture* 93: 87-94.

Fitzpatrick, M.S., B.K. Suzumoto, C.B. Schreck, and D. Oberbillig. 1984. Luteinizing hormone-releasing hormone analogue induces precocious ovulation in adult coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 43:67-73.

Garcia, L.Ma.B. 1989a. Development of an ovarian biopsy technique in the sea bass, *Lates calcarifer* (Bloch). *Aquaculture* 77:97-102.

Garcia, L.Ma.B. 1989b. Spawning response of mature female sea bass, *Lates calcarifer* (Bloch), to a single injection of luteinizing hormone-releasing hormone analogue: effect of dose and initial oocyte size. *J. Appl. Ichthyol.* 5:177-184.

Garcia, L.Ma.B. 1989c. Dose-dependent spawning response of mature female sea bass, *Lates calcarifer* (Bloch) to pelleted luteinizing hormone-releasing hormone analogue (LHRHa). *Aquaculture* 77: 85-96.

Glubokov, A.L., N.N. Motloch and M.A. Sedova. 1991. Effect of a synthetic LHRH analogue and dopamine antagonists on the maturation of bream, *Abramis brama* L. *Aquaculture* 95: 373-377.

Goff, G. 1993. Flatfish aquaculture research: an evaluation of the potential of small commercial flatfish for culture with emphasis in yellowtail flounder, *Pleuronectes ferruginea*. A Newfoundland Inshore Fisheries Development Agreement contract on Flatfish Aquaculture Research. 19p.

Harmin, S.A., L.W. Crim, and M.D. Wiegand. 1995. Plasma sex steroids and the seasonal reproductive cycle in male and female winter flounder, *Pleuronectes americanus*. *Mar. Biol.* 121:601-610.

Harmin, S.A. and L.W. Crim. 1992. Gonadotropic hormone-releasing hormone analog (GnRH-A) induced ovulation and spawning in female winter flounder, *Pseudopleuronectes americanus* (Walbaum). *Aquaculture* 104:375-390.

Harvey, B. J. Nacario, L.W. Crim, J.V. Juario and C.L. Marte. 1985. Induced spawning of sea bass, *Lates calcarifer*, and rabbitfish, *Siganus guttatus*, after implantation of pelleted LHRH analogue. *Aquaculture* 47: 53-59.

Harvey, B.J. and W.S. Hoar. 1979. Methods of ovarian biopsy. *In:* The Theory and Practice of Induced Breeding in Fish. IRDC, Ottawa, Ontario. pp.35-36.

Heming, T.A. and R.P. Preston. 1981. Differential effect of formalin preservation on yolk and tissue of young chinook salmon (*Oncorhynchus tshawytscha* Walbaum). *Can. J. Zool.* 59:1608-1611.

Howell, W.H. 1983. Seasonal changes in the ovaries of adult yellowtail flounder, *Limanda ferruginea*. *Fish. Bull. U.S.* 81(2):341-355.

Howell, W.H. and D.H Kesler 1977. Fecundity of the Southern New England stock of yellowtail flounder, *Limanda ferruginea*. *Fish. Bull. U.S.* 75:877-880.

Kjørsvik, E., A. Mangor-Jensen, and I. Holmefjord. 1990. Egg quality in fishes. *Adv. Mar. Biol.* 26:71-113.

Kuo, C.-M., C.E. Nash, and Z.H. Shehadeh. 1974. A procedural guide to induce spawning in grey mullet (*Mugil cephalus* L.). *Aquaculture* 3:1-14.

Lam, T.J. 1983. Environmental influences on gonadal activity in fish. p.65-116. *In*: W.S. Hoar, D. J. Randall and E.M. Donaldson (eds.). Fish Physiology, vol. 9B. Academic Press, New York.

Larsson, D.G.J., C.C. Mylonas, Y. Zohar, and L.W. Crim. 1997. Gonadotropin releasing hormone-analogue (GnRH-A) advances ovulation and improves the reproductive performance of a cold-water batch-spawning teleost, the yellowtail flounder (*Pleuronectes ferrugineus*). *Can. J. Fish. Aquat. Sci.* In press.

Linehan, R. 1996. Growth and reproductive responses of the yellowtail flounder (*Pleuronectes ferrugineus* Storer) to photoperiod manipulation. B.Sc (Hons.) thesis. Memorial University of Newfoundland, St. John's, Newfoundland. 35p.

Lux, F.E. and F.E. Nichy. 1969. Growth of yellowtail flounder, *Limanda ferruginea* (Storer), on three New England fishing grounds. *ICNAF Res. Bull.* 6: 5-25.

Manickam, P. and K.P. Joy. 1989. Induction of maturation and ovulation by pimozide-LHRH analogue treatment and resulting high quality egg production in the Asian catfish, *Clarias batrachus* (L.). *Aquaculture* 83: 193-199.

Manning, A.J. 1997. An investigation of batch-spawning reproduction in captive yellowtail flounder (*Pleuronectes ferrugineus*). M.Sc. Thesis. Memorial University of Newfoundland, St. John's, Newfoundland. 117p.

McLean, E., D.B. Parker, C.M. Warby, N.M. Sherwood and E.M. Donaldson. 1991. Gonadotropin release following oral delivery of luteinizing hormone-releasing hormone and its superactive analogue (des-Gly^{10}[D-Ala^{6}] LHRH ethylamide) to 17β-estradiol-primed coho salmon, *Oncorhynchus kisutch* (Walbaum). *J. Fish Biol.* 38: 851-858.

Mylonas, C.C., Y. Magnus, A. Gissis, Y. Klebanov, Y. Zohar. 1996. Application of controlled-release, GnRHa delivery systems in commercial production of white bass × striped bass hybrids (sunshine bass), using captive broodstocks. *Aquaculture*. 140(3): 265-280.

Mylonas, C.C., Y. Tabat, R. Langer and Y. Zohar. 1995. Preparation and evaluation of polyanhydride microspheres containing gonadotropin-releasing hormone (GnRH), for inducing ovulation and spermiation in fish. *J. Control. Release* 35: 23-34.

Mylonas, C.C., J.M. Hinshaw and C.V. Sullivan. 1992. GnRHa-induced ovulation of brown trout (*Salmo trutta*) and its effects on egg quality. *Aquaculture* 106: 379-392.

Peter, R.E., H.-R. Lin, G. Van der Kraak and M. Little. 1993. Releasing hormones, dopamine antagonists and induced spawning. p.25-30. *In*: J.F. Muir and R.J. Roberts (eds.). Recent Advances in Aquaculture, vol. 4. Blackwell Science, Oxford.

Peter, R.E., H.-R. Lin and G. Van Der Kraak. 1988. Induced ovulation and spawning of cultured freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. *Aquaculture* 74: 1-10.

Pitt, T.K. 1974. Age composition and growth of the yellowtail flounder (*Limanda ferruginea*) from the Grand Bank. *J. Fish. Res. Board Can.* 31(11):1800-1802.

Pitt, T.K. 1971. Fecundity of yellowtail flounder (*Limanda ferruginea*) from the Grand Bank, Newfoundland. *J. Fish. Res. Board Can.* 28(3): 456-457.

Pitt, T.K. 1970. Distribution, abundance, and spawning of yellowtail flounder, *Limanda ferruginea*, in the Newfoundland area of the Northwest Atlantic. *J. Fish. Res. Board Can.* 27:2261-2271.

Ramos, J. 1986a. Luteinizing hormone-releasing hormone analogue (LH-RHa) induces precocious ovulation in common sole (*Solea solea* L.). *Aquaculture* 54:185-190.

Ramos, J. 1986b. Induction of spawning in common sole (*Solea solea* L.) with human chorionic gonadotropin (HCG). *Aquaculture* 56:239-242.

Reimers, E., A.G. Kjorrefjord, and S.M. Stavostrand. 1993. Compensatory growth and reduced maturation in second sea winter farmed Atlantic salmon following starvation in February and March. *J. Fish. Biol.* 43(5): 805-810.

Royce, W.F., R.J. Buller, and E.D. Premetz. 1959. Decline of the yellowtail flounder (*Limanda ferruginea*) off New England. *U.S. Fish Wildl. Serv., Fish. Bull.* 59:169-267.

Sanborn, H.R. and D.A. Misitano. 1991. Hormonally induced spawning of English sole (*Parophrys vetulus* Girard). *J. Appl. Ichthyol.* 7(1): 15-25.

Scott, A.P., M. Htun Han, V.J. Bye, J.R.C. Springate, and S.M. Baynes. 1980. Effect of photoperiod on gonadal maturation of female dab *Limanda limanda* (L.). *Gen. Comp. Endocrinol.* 40: 336.

Scott, D.M. 1947. The biology of the yellowtail flounder (*Limanda ferruginea* Storer). M.Sc. thesis, McGill University. Fish. Res Bd. Can. Manuscript Reports of the Biological Stations #361. 90p.

Scott, W.B. and M.G. Scott. 1988. Atlantic Fishes of Canada. *Can. Bull. Fish. Aquat. Sci.* 219: 713p.

Shehadeh, Z.H., C.-M. Kuo, and K.K. Milisen.1973. Validation of an *in vivo* method for monitoring ovarian development in the grey mullet (*Mugil cephalus* L.). *J. Fish Biol.* 5:489-496.

Sherwood, N.M., L.W. Crim, J. Carolsfeld and S.M. Walters. 1988. Sustained hormone release. I. Characteristics of *in vitro* release of gonadotropin-releasing hormone analogue (GnRH-A) from pellets. *Aquaculture* 74: 75-86.

Short, C.W., L.W. Crim, and M.J. Morgan. 1996. The effects of stress on spawning performance and larval development in Atlantic cod, *Gadus morhua*. p.136. *In*: F.W.

Goetz and P. Thomas (eds.). Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish. Austin, Texas.

Slater, C.H., C.B. Schreck and D.F. Amend. 1995. GnRHa injection accelerates final maturation and ovulation/spermiation of sockeye salmon (*Oncorhynchus nerka*) in both fresh and salt water. *Aquaculture* 130: 279-285.

Smigielski, A.S. 1979. Induced spawning and larval rearing of the yellowtail flounder, *Limanda ferruginea*. *Fish. Bull.* 76(4):931-936.

Sokal, R.R. and F.J. Rohlf. 1996. Biometry: The Principles and Practices of Statistics in Biological Research. 3rd Edition. W.H. Freeman and Company. New York. 859p.

Takizawa, K., Y. Fujita, Y. Ogushi, and S. Matsuno. 1994. Relative change in body length and weight in several fish larvae due to formalin fixation and preservation. *Fish. Sci.* 60(4):355-359.

Tamaru, C.S., C.S. Lee, C.D. Kelley and J.E. Banno. 1988. Effectiveness of chronic LHRH-analogue and 17α-methyltestosterone therapy, administered at different times prior to the spawning season, on the maturation of milkfish (*Chanos chanos*). *Aquaculture* 70:159-167.

Taranger, G.L. S.O. Stefansson and T. Hansen. 1992. Advancement and synchronization of ovulation in Atlantic salmon (*Salmo salar* L.) following injections of LHRH analogue. *Aquaculture* 102:169-175.

Thomas, P. and C.R. Arnold. 1993. Environmental and hormonal control of reproduction in sciaenid fish. p.31-42. *In*: J.F. Muir and R.J. Roberts (eds.). Recent Advances in Aquaculture, vol. 4. Blackwell Science, Oxford.

Thomas, P. and N.W. Boyd. 1989. Dietary administration of an LHRH analogue induces spawning of spotted seatrout (*Cynoscion nebulosus*). *Aquaculture* 80: 363-370.

Tucker, J.W., Jr. and A.J. Chester. 1984. Effects of salinity, formalin concentration and buffer on quality of preservation of southern flounder (*Paralichthys lethostigma*) larvae. *Copeia* 4:981-988.

Wallace, R.A. and K. Selman. 1981. Cellular and dynamic aspects of oocyte growth in teleosts. *Amer. Zool.* 21:325-343.

Walsh, S.J., W.B. Brodie, D.B. Atkinson and D. Power. 1995. An assessment of the yellowtail flounder stock in Divisions 3LNO. NAFO SCR Doc 95/74: 44p.

Yevseyenko, S.A. and M.N. Nevinskiy. 1982. On the development of the eggs and larvae of the yellow-tailed dab, *Limanda ferruginea* Storer, and their passive transport in the Northwestern Atlantic. *J. Ichthyol.* 21(5):65-74.

Zamarro, J. 1991. Batch fecundity and spawning frequency of yellowtail flounder (*Limanda ferruginea*) on the Grand Bank. *NAFO Sci. Coun. Studies*, 15:43-51.

Zar, J.H. 1982. Biostatistical Analysis, 2nd Edition. Prentice Hall. New Jersey. 718p.

Zohar, Y., M. Harel, S. Hassin and A. Tandler. 1995. Gilt-head sea bream (*Sparus aurata*). p.94-117. *In*: N.R. Bromage and R.J. Roberts (eds.). Broodstock Management and Egg and Larval Quality. Blackwell Science, Cambridge, Ma.

Zohar, Y. 1989. Fish reproduction: its physiology and artificial manipulation. p.65-119. *In*: M. Shilo and S. Sarig (eds.). Fish Culture in Warm Water Systems: Problems and Trends. CRC Press, Inc., Boca Raton, Florida.

Zohar, Y., M. Tosky, G. Pagelson and Y. Finkelman. 1989. Induction of spawning in the gilthead sea bream, *Sparus aurata*, using (D-Ala^6, Pro^9 NET)-LHRH: comparison with the use of HCG. *Israeli Journal Of Aquaculture Bamidgeh* 41: 105-113.